Têxteis funcionais no tratamento da Dermatite atópica

Biofunctional textiles in Atopic Dermatitis management

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals I-VI:

I Soares J, Lopes C, Tavaria F, Delgado L, Pintando M. **A diversity profile from the staphylococcal community on atopic dermatitis skin: a molecular approach**. *J Appl Microbiol.* 2013 Dec; 115(6): 1411-9.

II Lopes C, Rocha L, Fernandes S, Soares J, Tavaria F, Pintado M, Sokhatska O, Moreira A, Delgado L. **Filaggrin polymorphism Pro478Ser relates with atopic dermatitis severity and Staphylococcal aureus colonization**. *J Investig Allergol Clin Immunol*.; 26(1), 2016

III Lopes C, Pinto L, Leite C, Delgado L, Moreira A, Lourinho I. Personality traits may influence atopic dermatitis severity in adult patients: pilot study *J Investig Allergol Clin Immunol* (*in press*)

IV Lopes C, Silva D, Delgado L, Correia O, Moreira A. **Functional textiles for atopic dermatitis: a systematic review and meta-analysis** *Pediatr Allergy Immunol.* 2013 Sep; 24(6):603-13

V Lopes C, Soares J, Tavaria F, Pintado M, Duarte AF, Correia O, Delgado L, Moreira A. **Chitosan coated textiles may improve atopic dermatitis severity by modulating skin staphylococcal profile: a randomized controlled trial**. *PLoS One*. 2015 Nov 30;10 (11)

VI Lopes C, Sokhatska O, Moreira A, Delgado L. Chitosan coated textiles increase serum eosinophil cationic protein but not specific IgE in atopic dermatitis patients: a randomized controlled trial *(submitted)*

ABBREVIATIONS

AD	atopic dermatitis
ANCOVA	analysis of covariance
CFU	colony forming unit
COS	chitosan oligosacharides
DCS	dendritic cells
DD	degree of deacetylation
DLQI	Dermatology Life Quality Index
ECP	eosinophil cationic protein
EVOH	Ethylene Vinyl Alcohol Fibre
FLG	Filaggrin
GRADE	Grading of Recommendations Assessment, Development and Evaluation
HSV	herpes simplex virus
IL	Inter-leukine
ITT	Intention-to-treat
MW	molecular weights
NEO-PI-R	NEO personality inventory
PMN	polymorphonuclear
QoL	quality of life
SAGS	Superantigens
SCORAD	Scoring atopic dermatitis index
SE	staphyloccal enterotoxin
sTNF	soluble receptor tumor necrosis factor
sIgE	Specific IgE
TEWL	Transepidermal water loss

ABSTRACT

Allergic diseases represent a global health problem with increasing prevalence, mostly in developing countries. Recent theories related this increment with loss of microdiversity (saprophytes micro-organisms from the environment or endogenous) that are essential to barrier and immunologic tolerance maintenance.

Atopic dermatitis (AD) is one of the clinical expressions of allergic disease. It is an inflammatory skin disorder characterized by exacerbations and remission of intensely pruritic lesions of variable location. It affects predominantly children, but when persisting in adolescence and adulthood, tends to be more severe. It can precede or coexist with other allergic manifestations as asthma and food allergies.

Host and environmental factors contribute to its pathogenesis and manifestations. The former include genetic background, namely Filaggrin gene mutations, innate and adaptive immunological dysfunction and psychological aspects that interfere wit patients quality of life. Environmental factors include allergens and skin microbiome that can modulate expression and severity of AD.

In parallel with its etiopathogeny, AD treatment is multidimensional, aiming at restoring skin hydration, downregulating skin inflammation, refraining pruritus and treating clinical infection. Functional textiles (textiles with applications in the medical field) have recently emerged as complementary to conventional treatment not only by improving skin comfort and diminishing symptoms, but also by its potential regulatory role of skin microbiological profile. Chitosan, a carbohydratepolymer with antiseptic and immunomodulatory properties already in use for treatment of skin wounds and burns, has been considered potentially useful in AD management.

This thesis aims to investigate the influence of skin microbiologic, genetic, immunoallergic and psychological factors in atopic dermatitis and the impact of a chitosan coated textile in its management.

The thesis is based on three types of studies: 1) cross sectional analysis of AD patients and controls assessing the diversity of skin staphylococci community, of AD patients assessing the relation between filaggrin mutations, skin microbiologic profile serum allergic markers and clinical severity; and the association between psychological factors, disease severity and quality of life; 2) a systematic literature review and meta-analysis of studies that have used functional textiles to manage AD; 3) a randomized controlled trial assessing the efficacy and safety of chitosan coated garments and its immunoallergic effects. A total of 78 subjects participated in the randomized controlled trial and 107 in the cross sectional surveys.

When compared to healthy subjects, the skin staphylococcal community of AD patients determined by a novel multiplex PCR was less diverse, with predominance of *S. epidermidis* and *S. aureus* that harboured in their majority, genes encoding superantigens. The filaggrin loss of function mutations R501X and 2282del4 were not associated with AD severity, bacterial colonization nor allergic systemic

markers in contrast with the polymorphism P478S that was associated with more severe disease and increased *S. aureus* colonization. Based on personality traits, anxiety and depression questionnaires, higher scores on personality trait consciousness, meaning more self-control, were associated with lower disease severity in contrast with depressive symptoms. In AD patients, the use of different functional textiles were associated with distinct effects: silver-coated fabrics seemed to be more effective at diminishing the severity of lesions, while silk fabrics seemed to perform better in terms of alleviating pruritus and other symptoms. Results from the clinical trial showed that chitosan coated garments used during the night for 8 weeks as pyjamas were safe, may impact on AD severity by modulating the skin staphylococcal profile and that a potential impact on quality of life may be considered. Additionally, an immunomodulatory effect may occur linked to eosinophilic activation, but the clinical meaning of this finding remains unknown. Our study is the first showing, that having an effect on skin microbiome, dermatitis clinical symptoms may be modified. More studies are needed before a recommendation regarding the use of chitosan coated garments in AD can be made.

RESUMO

As doenças alérgicas representam um problema global de saúde e a sua prevalência tem vindo a aumentar principalmente em países em vias de desenvolvimento. Teorias recentes relacionam este aumento da prevalência com a perda de microdiversidade (microrganismos saprófitas do meio ambiente ou do self) que são essenciais no desenvolvimento e manutenção das barreiras e da tolerância imunológica.

A Dermatite atópica é uma da manifestações da doença alérgica. É uma doença cutânea inflamatória crónica caraterizada por períodos de exacerbação e remissão de lesões intensamente pruriticas e de localização variável. Afecta sobretudo as crianças mas quando perdura na adolescência e idade adulta tende a ser mais grave. Pode preceder ou coexistir com outras cormorbilidades alérgicas como asma e alergia alimentar.

Para a sua patogénese e expressão contribuem factores do hospedeiro e do ambiente. Os primeiros incluem factores genéticos, nomeadamente a presença de mutações do gene da filagrina e disfunção imunológica inata e adaptativa, e aspectos psicológicos que interferem com a qualidade de vida. Nos factores ambientais podemos incluir os alergénios e microbioma cutâneo que podem modular a expressão e gravidade da DA.

Em paralelo com a etiopatagenia, o tratamento é também multifacetado: tem por objectivo a hidratação da barreira cutânea, diminuição da inflamação, do prurido e tratamento da infecção. Os têxteis funcionais(têxteis com aplicações na área médica) surgiram como complementares ao tratamento convencional não só por aumentarem o conforto e poderem diminuir os sintomas mas também pela possibilidade de regularem o perfil microbiológico da pele. O quitosano, um biopolímero de carbohidrato com propriedades antissépticas e imunomoduladoras foi considerado potencialmente útil no tratamento de doentes com Dermatite atópica.

Assim, esta tese tem por objetivo investigar a relação entre o perfil microbiológico cutâneo, factores genéticos, imunoalérgicos e psicológicos em doentes com DA; e avaliar o impacto de um têxtil com quitosano no seu tratamento.

Esta tese é baseada em três tipos de estudos: 1) estudo transversal de doentes com DA avaliando a diversidade da comunidade estafilocócica cutânea e a relação entre mutações e polimorfismos do gene da filagrina, perfil microbiológico e marcadores de inflamação alérgica; associação entre factores psicológicos, gravidade da DA e qualidade de vida 2) revisão sistemática e meta-analise da literatura dos estudos que incluíram têxteis funcionais no tratamento da DA 3) estudos randomizados avaliando a eficácia e segurança e efeitos imunomoduladores de têxteis impregnados com um novo biopolimero, o quitosano. Um total de 78 doentes participaram no estudo randomizado controlado e 107 nos estudos transversais .

Quando comparado com indivíduos saudáveis, a comunidade estafilocócica cutânea de doentes com DA é menos diversa, com predomínio de *S. epidermidis* e *S. aureus.* As mutações R501X e 2282del4

do gene da filagrina não se associaram a SCORAD mais elevado, maior colonização microbiana ou inflamação alérgica mas em contrapartida, o polimorfismo P478S associou-se a maior gravidade e maior colonização por *S.aureus* . Baseado em questionários de personalidade, ansiedade e depressao, scores mais elevados no traço conscienciosidade, significando mais autocontrolo estiveram associados a doença menos grave em contraste com sintomas de depressão. Na Dermatite atópica, o uso de diferentes têxteis funcionais foram associados a efeitos distintos: têxteis com sais de prata parecem ser mais eficazes a diminuir a gravidade das lesões, enquanto os têxteis com seda parecem ser mais úteis no alivio do prurido cutâneo. Baseado em evidência de baixa qualidade acerca da eficácia destes têxteis funcionais, a força de recomendação para a sua utilização é fraca. Os resultados do ensaio clinico sugeriram que a utilização de têxteis com quitosano pode ter impacto na gravidade da doença modulando o perfil estafilócico cutâneo e que um potencial efeito na qualidade de vida deve ser considerado. Adicionalmente pode ocorrer um efeito imunomodulador relacionado com a activação eosinofilica mas cujo significado clinico é desconhecido. Trata-se do primeiro estudo que indica que uma intervenção no microbioma cutâneo poderá modificar os sintomas clínicos de Dermatite atópica. Mais estudos serão necessários antes de se poder formular uma recomendação da sua utilidade nesta patologia .

1. INTRODUCTION

Atopic disorders represent a global health problem. A number of studies have demonstrated an increase in the prevalence of asthma, allergic rhinitis and atopic dermatitis (AD) over the last four decades (1). Although current indications point to AD symptoms having leveled off or even having decreased in some countries such as the United Kingdom and New Zealand (2), it remains a serious health concern in many countries, and particularly in the developing world, where the disease is still very much on the rise (3).

The sharp increase in allergic diseases between early 1960s and late 1980s is perceived to be a consequence of an intense migration from rural to urban regions, from poor, developing countries to rich, but heavily industrialized regions of Europe, Asia and the Americas. The recent biodiversity hypothesis and allergic diseases (4) claims that not only the loss of macrodiversity determined by climate change and pollution is associated with adverse health effects, but the loss of microdiversity is also associated with various inflammatory conditions, including asthma and allergic diseases. A fundamental role for microorganisms in human health, whether indigenous or environmental, is becoming increasingly evident.

Commensals are no longer considered as passive bystanders or transient passengers, but increasingly as active and essential participants in the development and maintenance of barrier function and immunological tolerance (5). They are also involved in the programming of many aspects of T cell differentiation in co-operation with the host genome (6) and mounting evidence also showed that alterations in the indigenous microbiota correlated with inflammatory disease states (7). After gut and lung microbiota characterization, the study of skin microbiome, comprising the diverse and complex microbial ecosystems inhabiting the skin is increasing. The possible role of staphylococci in predisposing to AD has become a good example of the complex interaction between skin microbiologic profile and an inflammatory skin condition (7). Besides the importance of the environment, the increased familiar predisposition to develop allergic diseases also raised the hypothesis that host genetic factors could be involved in AD pathogenesis.

The discovery, in 2006, that loss-of-function mutations in the filaggrin (FLG) gene were a strong genetic risk factor for AD, marked a significant breakthrough. FLG monomers aggregate keratin filaments into tight bundles, resulting in collapse and flattening of corneocytes maintaining skin barrier integrity, normal stratum corneum lipids and lowering skin pH (8). Therefore, the mutations of the FLG gene may increase skin permeability, predisposing to allergen penetration and skin infection. More recently, some single-nucleotide polymorphisms (SNP) have also been shown to increase susceptibility to develop AD and subsequent colonization with Staphylococci species especially in asiatic populations. Nevertheless, the fact that aapproximately 10% of Northern European subjects from the general population are heterozygous mutation carriers(9), and that some patients seem to outgrow their disease (10) show that FLG mutations and SNP are not the only cause of AD and that

there may be a close relation between the genetic, skin microbiological and immunological status in AD .

Innate and adaptive immune dysfunction are typical features of AD. Atopic skin exhibits decreased levels of antimicrobial peptides, and decreased number of dendritic cells when compared to the skin of patients with other inflammatory skin diseases. A TH2-dominated cytokine milieu downregulates the antimicrobial peptidic response in AD skin and FLG expression in keratinocytes . AD patients have increased risk of developing rhinitis and asthma suggesting a systemic Th2- allergic predisposition. The connection between skin barrier dysfunction, skin microbiome profile, innate and adaptive immune deregulation seems therefore to close a deleterious vicious cycle.

Psychological aspects contribute not to AD pathogenesis but as potential exacerbating factors. Stress can perturb epidermal permeability (11, 12) promoting the release of neuropeptides such as nerve growth factor, neurotensin, calcitonin gene-related peptide and substance P-that are pruritogenic and proinflammatory mediators. However, the impact of stressful events on the individual may be modulated by personality. AD patients have been described as being more neurotic, hostile, anxious and depressive when compared to healthy controls (13), but few studies have addressed this relation with disease severity in a real life setting. Consequently, the treatment of AD should be multidimensional, embracing all the factors contributing to its pathogens and exacerbation.

AD management is based on pharmacological approaches aimed at diminishing pruritus, immunosuppressing inflammation and treating skin infection. Non-pharmacological strategies include the restoration of skin hydration and psychological interventions (14). Textiles are an important part of AD management since, depending on their tactile, thermic and physical properties, they can exacerbate or improve pruritus. Fabrics such as cotton and silk garments are usually recommended due to their tendency to reduce scratch and aid in emollient absorption (15). With the development of nanotechnology, intelligent or functional textiles, incorporating new biopolymers, have been designed with beneficial effects on human health(16). One of the new biopolymers with suitable characteristics due to its low immunogenicity is chitosan. Chitosan is derived from chitin found mainly in crustaceans, molluscs, marine diatoms, insects, algae, fungi and yeasts. Chitosan textiles had already been used as adjuvants and antiseptic dressings in burns and wound healing with promising results (17, 18). In immunologically mediated skin diseases, and AD in particular, the clinical utility of chitosan-coated textiles is not known.

This study aims to investigate the relation between skin microbiologic, genetic, immunoallergic and psychological factors in AD and the impact of a chitosan coated textile in its management.

2. REVIEW OF LITERATURE

2.1 Atopic dermatitis

2.1.1 Definition and epidemiology

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by exacerbations and remission of intensely pruritic lesions of variable location. It affects predominantly children, but tends to be more severe when persisting in adolescence and adulthood (19). Studies in the first half of the twentieth century have shown an incidence of 2%–3% (20), while more recent surveys have shown an increase to 9%–12% in childhood. Its prevalence has been found unevenly distributed over the world: on 6-7 years old ranged from 0.9% in India to 22.5% in Ecuador, with new data showing high values in Asia and Latin America (2). For the age group 13 to 14 years, prevalence values ranged from 0.2% in China to 24.6% in Columbia with the highest values in Africa and Latin America. In Portugal, it was found to be 9.3 % and 5.2% on 6-7 and 13-14 years old respectively (21).

2.1.2 Pathogenesis

Allergic diseases and the biodiversity hypothesis

Biodiversity can be broadly defined as the variety of life on Earth. It includes the genes in all living cells, populations, species and their communities, the habitats in which they occur, and the ecosystems they comprise. The Biodiversity Hypothesis proposes that reduced contact of people with natural diverse environments, including environmental microbiota, adversely affects the assembly and composition of human commensal microbiotas and may thereby lead to inadequate stimulation of immunoregulatory circuits and ultimately to clinical disease .

Previous studies reveal that microbe-rich environments confer protection against allergic and autoimmune diseases (22), but it is likely that declining biodiversity is more generally responsible for human immune dysfunction. Hanski et al. (23) showed recently, that compared with healthy adolescents the atopic individuals had lower environmental biodiversity – in the form of species richness of native flowering plants and in the land use type – in the surroundings of their homes. The atopic adolescents also had significantly lower generic diversity of Gram-negative gammaproteobacteria on their skin. Furthermore, the abundance of the genus Acinetobacteria on the skin was positively correlated with the peripheral mononuclear cell expression of IL-10, a key anti-inflammatory cytokine in immunological tolerance. Gammaproteobacteria are common in the soil, but are particularly dominant in aboveground vegetation, such as flowering plants.

Modern research focuses on microbiotas inhabiting the barriers of man and environment. The genetic composition of the barrier microbiotas, microbiomes mediate the signalling between human DNA and

environmental DNA. It is this cross-talk, which we are only starting to explore, that determines our survival; the capability of the human immune system to distinguish between danger and non-danger, and the difference between self and non-self. The barrier microbiomes can be regarded as the "second genetic reservoir" of man, co-existing as a result of co-evolution of millions of years. Pressure caused by the ever growing human populations has direct effects through habitat destruction and indirect effects through climate change (24). Climate change has the potential to increase aeroallergens such as pollen and mold spores by earlier start of pollen season, increased allergenicity, and changes in pollen spatial distribution. These changes adversely impact allergic diseases.

The biodiversity hypothesis can be regarded as an extension of hygiene (25) or "old friends" hypothesis (26)and microbial deprivation or microbiota hypothesis (27). Population growth (urbanization) leads to loss of biodiversity (poor macrobiota/microbiota), poor human microbiota (dysbiosis), immune dysfunction (poor tolerance), inflammation and finally to clinical disease.

Skin microbiome in AD patients

Recent metagenomic studies have revealed that diverse and complex microbial ecosystems inhabit the skin, collectively known as the skin microbiome (28). The skin microbiota is composed mainly of members of the same four phyla that comprise the gut microbiota, although with dissimilar relative abundances (29). In all individuals, Propionibacterium species dominate sebaceous areas such as the forehead, retroauricular crease, and back, whereas Staphylococcus and Corynebacterium species dominate moist areas, such as the axillae and abundant Gram-negative organisms, previously thought to colonize the skin rarely as gastrointestinal contaminants, were found in the microbiomes of dry skin habitats, such as the forearm or leg (30).

In recent years, the relation between AD and metagonomics has raised increased interest. Previous studies showed that Staphylococcus species increased from 35% to 90% of the microbiome during flareups, and surprisingly, with concomitant increase of *S.epidermidis* (31). It is still unclear whether *S. aureus* and *S. epidermidis* mutually enhance each other's colonization or whether *S.epidermidis* increases as an antagonistic response to an increasing *S.aureus* population. *S.aureus* also produce superantigens (S. enterotoxin A, S. enterotoxin B and C, toxic shock syndrome toxin-1), which are important effectors in AD (32). They cause *S.aureus* -specific IgE production that correlates with disease severity (33). Superantigens also cause nonspecific IgE production, activate T cells, B cells, and macrophages, and stimulate their proliferation (34). Lately, they have been found to induce chemokines such as CCL1 and CCL18, which bind to CLA-positive T cells in peripheral blood, thus possibly playing a role in T cell homing to the skin (35). The superantigens seem to reduce the immunosuppressive activity of certain immunosuppressive regulatory T cells, which may, in turn, increase the inflammatory T cell activation(34). They are also known to induce corticosteroid resistance complicating the treatment of atopic diseases (36).

Besides pathogenic bacteria, virus and fungi can also cause infection in AD patients. Infection with Herpes simplex virus (HSV) infections can lead to the acute disseminated viral infection eczema herpeticatum, which often requires hospitalization (37). Malassezia yeast species colonize the skin of

90% of AD patients compared with 35% of healthy controls, especially the sebaceous areas face, scalp and upper body. Species associated with AD include *Malassezia globosa, sympodialis, restricta,* and *furfur* (38) Their role in AD exacerbations has been controversial despite the fact that specific IgE antibodies to *Malassezia* species can be found in AD patients but not in healthy controls (39).

Skin barrier defects and filaggrin mutations

Dryness of the skin is a hallmark of patients with AD. It is due to a defect in the epidermal barrier and as a consequence, an increased transepidermal water loss. Appropriate function of skin barrier is secured by an interplay of proteins of the keratin cytoskeleton (eg, filaggrin (40), involucrin, and loricrin), of intercellular lipids (eg, ceramides) provided by keratinocyte-derived lamellar bodies, and of a set of epidermal proteases, such as the stratum corneum chymotryptic enzyme (41). FLG is a key protein of epidermal differentiation serving as a template for the assembly of the cornified envelope. Its breakdown products critically contribute to the water-binding capacity of the stratum corneum.

To date, 20 *FLG* mutations have been identified in European populations. In Asian populations, an additional 17 mutations, of which eight are prevalent and nine occur at a low frequency, have been identified(10) . *FLG* is located within the epidermal differentiation complex on chromosome 1q21, a dense cluster of genes involved in the terminal differentiation of the epidermis and the formation of the stratum corneum (42). The prevalence of FLG-null mutations varies across Europe, but R501X and 2282del4 are the two most common mutations and they have consistently shown significant association with AD in the European continent, with the one exception of the Italian population (43). R501X and 2282del4 are rare in Italian AD cases (allele frequency of 1% for each) (44), and full sequencing of FLG exon 3, exon 2, and the promoter region in a total of 220 Italian atopic dermatitis patients identified only three additional mutations and no association with AD (45). The pattern of FLG mutations in other Mediterranean populations has not yet been examined, but the Italian data suggest that different genetic factors may predispose to AD in these populations warranting further investigation.

Single-nucleotide polymorphism (SNP) of FLG gene have also been studied mainly in Asian population. *FLG* P478S SNP is the most common variant of the *FLG* coding region in the SNP database of the NCBI10 and may serve as a good screening tool of AD. It encodes either proline (CCT) or serine (TCT) and was found to be associated with AD and psoriasis in Chinese and Taiwanese populations. There is no data concerning its prevalence or clinical significance in European population.

In recent years, the hypothesis that mechanisms other than FLG mutations can contribute to skin barrier impairment has emerged. The levels of FLG and its degradation products seem to be influenced by inflammation and exogenous stressors. Environmental factors such as low humidity (46), psychological stress (47), and inflammatory cytokines as IL-4, IL-13, IL-17A, IL-22, IL-25, IL-31, TNF-alpha (48) seem to reduce its levels independently of FLG mutation status. Moreover, the fact

that at least 50% of all patients with AD do not show any FLG mutations and that even those who have mutations grow out of their disease, (3) indicates that defects in barrier proteins other than FLG could contribute to barrier dysfunction in AD and compensatory mechanisms must be operative to restore a normal skin barrier function.

Immunological dysregulation

Innate immune dysfunction potentially plays an important role in AD. Ample evidence now exists that epithelial cells from atopic skin exhibits decreased levels of antimicrobial peptides, including defensins, cathelicidins, dermicidin and psoriasin (49). Plasmacytoid dendritic cells (DCs), an important component of the innate response, are in decreased number in the skin of patients with AD when compared with the skin of patients with other inflammatory skin diseases, such as psoriasis, contact dermatitis or lupus erythematosus (50). This fact may explain the increased risk of infection with certain types of bacteria (*S.aureus*), viral (herpes simplex virus and pox viruses), and fungal (*Malassezia sympodialis*) infections (51).

T cell response may also take part in AD pathogenesis. Th2 polarization is characteristic of acute lesions in contrast with chronic lesions in which there seems to exist a Th1 skewing. It appears that both Langerhans cells and inflammatory DCs are important in this regard: the former probably contribute to the TH2 polarization, the latter seems to be responsible for deviation of the immune response in the TH1 direction (52). Recent evidence exists that eosinophil- and basophil-derived IL-25, a distinct member of the IL-17 cytokine family, enhances the expansion and functions of TH2 memory cells, thus augmenting allergic tissue inflammation (53). In keeping with these observations is the finding of substantial numbers of TH17 cells in acute, but not chronic, AD lesions, (54) indicating that IL-17 and IL-22 play important roles in the emergence of acute AD skin lesions. The factors responsible for the switch from a TH2/TH17- to a TH1-dominated allergic tissue response are not fully understood.

Dermal fibrosis is a salient feature of chronic AD lesions. There exists evidence that TGF-b and IL-11, mainly produced by eosinophils, are the major fibrogenic cytokines in chronic AD and that type I collagen is the major collagen subtype involved in this tissue-remodeling process.

Although a wide variety of biologic response modifiers (eg, neuropeptides, proteases, and kinins) can induce **pruritus**, the nature of the pathophysiologically relevant itch mediator has remained enigmatic. It appears that the TH2 cytokine IL-31 could be a major factor in this regard (55). It is significantly overexpressed in pruritic versus nonpruritic AD skin lesions and in leukocytes from atopic individuals compared with those from healthy control subjects.

Psychological factors in AD

Psychological factors and its relation with allergic diseases has long been a matter of concern (56). Distress has been considered, a relevant exacerbating factor of AD (57, 58) and personality traits modulate the coping strategies with emotions, interfering with the way patients experience their

disease (59). AD patients have been described as being more neurotic, hostile, anxious and depressive when compared to healthy controls (13). Moreover, in a recent study, specific personality traits as agreeableness predicted pruritus in an experimental setting (60). Nevertheless, there are no studies relating personality with disease severity or quality of life in AD patients in a real-life environment.

Regarding psychological symptoms, depressive and anxiety seem to be more common in adolescent and adults with AD; and its occurrence is related to objective and subjective assessment of disease severity (61). Besides personality and "psi" symptoms, quality of life (QOL), defined as the attitude by which an individual senses and reacts to his/her health condition and to other non-medical aspects of his/her life (62), constitutes an important patient oriented outcome in chronic diseases. The impact of personality and psychological distress in QOL is controversial (63-65).

With the growing prevalence of AD in developing countries and evidence of the psychosocial burden of this disease, identifying specific psychological patterns in defined populations will be relevant for the development of targeted assessments and treatment interventions.

Allergic versus non –allergic atopic dermatitis

About 70 to 80% of patients with AD are considered to have classical, i.e. IgE associated or allergic, AD because they show elevated sIgE levels or positive skin prick test results for aeroallergens or food allergens, whereas the remaining 20 to 30% never show this kind of IgE- mediated sensitization and are considered to have non-IgE associated or nonallergic AD (66). IgE-mediated sensitization may not yet be evident in infants or young children but it develops with increasing age. After a thorough or repeated allergologic work-up, some nonallergic AD patients may be reclassified as having allergic AD (37). It is unclear whether the allergic and nonallergic atopic diseases are truly separate diseases or represent different degrees of severity of the same disease. The nonallergic type is usually clinically milder and has a lower risk for asthma and allergic rhinitis and is characterized by less eosinophilia and fewer CLA-positive T cells as well as Fc **ε** RI-positive cells (37). Recent findings suggest that the nonallergic AD is associated with sensitization to microbial antigens such as *S. aureus*, *Malassezia* species (Piyrosporum), and *Candida albicans* (67), an association also seen in the allergic type.

2.1.3 Clinical features and diagnosis

The criteria established by Hanifin and Rajka have become the standard for the clinical diagnosis of AD (68). Acute lesions are characterized by erythematous papules, papulovesicles or weeping skin lesions. Subacute lesions reveal erythematous scaling papules and plaques, while chronic lesions consist mainly of lichenification in classically affected body areas. The localization of the lesions varies with age.

In early infancy, areas most affected include the scalp, the face (especially the cheeks and the chin),

the trunk and the extensor surfaces of the extremities. The diaper area as well as the nose are commonly spared in AD. The skin might be highly inflammatory, with large exsudative areas leading to formation of crusts. Lichenification is only rarely observed in early infancy, and itch might not be as debilitating as later in life. Non-erythematous areas are very often characterized by an intense dryness of the skin. Atopic shiners such as Dennie-Morgan infraorbital folds are observed most often in these patients. Infra-auricular fissures are also commonly seen. In childhood, skin lesions in the flexural areas such as the antecubital and popliteal fossae and the neck, as well as the wrists and the ankles, characterize AD. Lesions of the face are most often not as prominent as in infancy. These localizations tend to persist in adolescents and adults. Patients in this age range might be especially affected with lesions involving specific areas such as the perioral or the periocular areas. Generalized erythrodermia might be observed in a few patients with very severe recurrent lesions

The European Task Force established the SCORAD index on AD (1993) (69). It is composed of three different domains (A= extension B= intensity C = subjective symptoms). To determine extent, the sites affected by eczema are shaded on a drawing of a body. The rule of 9 is used to calculate the affected area (A) as a percentage of the whole body: Head and neck 9% Upper limbs 9% each , Lower limbs 18% each , Anterior trunk 18% ,Back 18% 1% each for genitals, each palm and the back of each hand. The score for each area is added up. The total area is 'A', which has a possible maximum of 100%. A representative area of eczema is selected. In this area, the intensity of each of the following signs is assessed as none (0), mild (1), moderate (2) or severe (3), Redness, Swelling, Oozing / crusting Scratch marks , Skin thickening (lichenification), Dryness (this is assessed in an area where there is no inflammation). The intensity scores are added together to give 'B' (maximum 18). Subjective symptoms i.e., itch and sleeplessness, are each scored by the patient or relative using a visual analogue scale where 0 is no itch (or no sleeplessness) and 10 is the worst imaginable itch (or sleeplessness). These scores are added to give 'C' (maximum 20). SCORAD was calculated by: extent/5+3.5xintensity+subjective symptoms (max.103). Extent and subjective symptoms each account for 20% of total score, and intensity accounts for the remaining 60%.

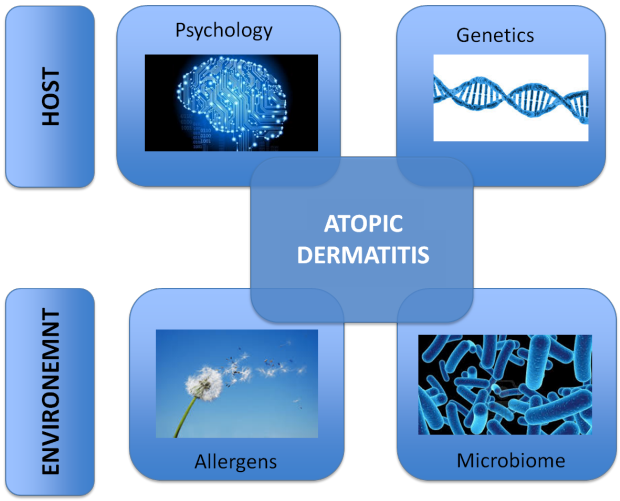

Figure 1 *Open framework-Atopic Dermatitis model*

2.1.4 Treatment

Pharmacological

Antihistamines are widely used for patients with atopic dermatitis, especially for pruritus, and also for co-existent allergic rhinoconjunctivitis and allergic asthma. Second- and third- generation antihistamines, such as cetirizine, levocetirizine, loratadine, desloratadine, and ebastine, are effective against pruritic immediate allergic reactions and urticaria. These compounds have effects on many mediators of inflammation and can be assumed to exert effects in the acute phase of AD (70).

Topical glucocorticosteroids (corticosteroids) have been the mainstay of the AD treatment for the last few decades. Their most important anti-inflammatory effects are inhibition of vasoactive substances such as kinins, histamine, prostaglandins, leukotriens, and complement. They reduce the permeability of cell membranes, and inhibit migration of leucocytes and macrophages, as well as inhibit serum extravasation and edema by reducing the permeability of the vascular endothelium. They are also antipruritic (71). Topical corticosteroids are officially indicated only for short-term use and are usually used for only 1 to 3 weeks. They relieve the symptoms and inflammation of AD quickly, but after the treatment period the disease is likely to relapse.

There are situations when topical corticosteroids may not be appropriate or must be discontinued due to risk or adverse effect. The **topical calcineurin inhibitors** represent another class of anti-inflammatory medications that can be used similarly but without the same adverse effect profile. Tacrolimus and pimecrolimus inhibit the activation of key cells involved in AD as T cells, dendritic cells, and mast cells(72). There is abundant clinical data that these medications are safe and effective in

treating AD in patients 2 years and older. Proactive therapy has been defined for these medications: twice weekly application to eczema-prone areas has been shown to reduce flares(73).

Systemic glucocorticosteroids such as prednisone, prednisolone, and methylprednisolone often serve as rescue therapy in severe AD exacerbations. In addition to having the same side-effects as topical corticosteroids, they may cause arterial hypertension, electrolyte imbalance, impaired glucose metabolism, Cushing's syndrome, and osteoporosis, especially in long-term treatment (70).

Systemic long-term immunosuppressive treatments are needed in severe AD. Cyclosporine for AD is extensively studied and has shown good efficacy in adults and children (74) (75). Azathioprine is an old compound used for similar indications as for cyclosporine. Until lately, randomized, controlled studies on its efficacy and safety for AD have been few (76, 77). Methotrexate is an old drug compound used similarly to azathioprine (78).

Other newer pharmacological treatment modalities tried for severe treatment-resistant AD include mycophenolate mofetil, interferon gamma (IFN- **γ**), intravenous immunoglobulin (IVIG) with conflicting results (79). Anti-immunoglubulin E (omalizumab), which binds to free IgE and membrane-bound IgE on B cells, has shown efficacy in allergic respiratory disease. Few studies with controversial results exist on its efficacy on AD (80-82), but larger studies are lacking.

Treatment of skin infections

Infected AD exacerbations require specific treatment of microorganisms in combination with eczema treatment, but no evidence supports the assumption that antimicrobial treatment of colonized skin will benefit patients in the long-term (83). Combining topical antibiotic agents with anti-inflammatory treatment has led to no further decrease in *S. aureus* colonization (84).Neither is there evidence that antifungal treatment's reducing *Malassezia* colonization would relieve AD in the long-term, although treatment periods with an antifungal agent have had some effect, especially on eczema of the sebaceous areas (85).

Non-Pharmacological

UV-light treatment is suitable for patients whose AD improves during sunlight exposure in the summer time, but not for UV-sensitive patients. Narrow-band UVB treatment is the most common and is usually suitable as concomitant therapy with topical treatment or as maintenance therapy (86).

Considering the importance of saprophytes in gut microbiome various studies have tried to endorse tolerance with supplementation of **probiotics**. The rationale of its use was to improve mucosal health by their ability to strengthen tolerance against e.g. allergens, and on the other side, improve mucosal defence system against harmful pathogens (87). Accumulating data showed that probiotics supplementation efficacy in AD prevention and management is controversial (88).

2.2 Functional textiles on atopic dermatitis

Textiles are considered an important part of AD management, and fabrics such as cotton and silk

garments tend to reduce scratching and aid emollient absorption (15). With the development of nanotechnology, intelligent, or functional, textiles, which are designed to have beneficial effects on human health, have emerged (16). Such textiles have been used as adjuvants and antiseptic dressings in burns and wound healing with promising results (17, 18). In immunologically mediated skin diseases, and AD in particular, the focus has been to improve itch sensation, severity of lesions, and skin colonization by *S. aureus*.

Most of the studies of functional textiles in AD have investigated the use of specially treated long-sleeved shirts and pants in close contact with the skin (Table 1). Cotton textiles can be functionalized with antiseptic silver salts (89, 90) or borage oil, which supplies fatty unsaturated acids to the skin barrier (91). Silk coated with specific antimicrobial chemical compounds and smooth ethylene vinyl alcohol (EVOH) fibres are also used to diminish physical stimuli applied to the skin (92). Nonetheless, contact between bioactive compounds in functional textiles and a disrupted skin barrier raises safety concerns, although the few studies addressing the potential risks of sensitization, disturbance of the ecology of the skin, and toxic side effects have shown functional textiles to be safe and usable (93).

Table 1 *Classification of functional textiles according to active compounds*

Functional textile	Textile Composition	Type of fabric	References
Silver	Silver loaded cellulose fabric with incorporated seaweed	Long sleeved shirts and leggings	(94),(95)
	Silver coated nylon fibres	Long sleeved shirts and leggings	(93)
	Silver coated to nylon fibres and polyamide	Long arm undershirts and pants for adults, whole body clothes for children	(89, 90)
Borage oil	Borage oil chemically bonded to cotton fibres	Undershirts	(91)
Ethylene Vinyl Alcohol Fibre	Alternately arranged hydrophilic and hydrophobic nanoscale segments	Underwear	(92)
Silk	Sericin free-silk treated with AEGIS/AEM 5772/5	Tubular sleeves	(96-98)
		Whole body romper suites, long sleeved T shirts, panty hoses	(99)
	Microair Sericin free-silk treated with AEGIS/AEM 5772/5	Body suits, rompers, leggings, tubular bands, gloves, waist bands	(100)
	Silk like 50% polyester and 50% nylon	Bedsheets	(101)

2. 2. 1.Chitosan

Chitin is the second most abundant natural polysaccharide on Earth following cellulose. It is found mainly in crustaceans, molluscs, marine diatoms, insects, algae, fungi and yeasts. It is a

polysaccharide composed of b-(1->4)-linked N-acetyl-D-glucosamine (GlcNAc) residues with an acetamide group at the C2 position. Partial deacetylation of chitin lead to chitosan that is a copolymer of glucosamine (b(1–4)-linked 2-amino-2- deoxy-D-glucose) and N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose).

Chitosan is, in fact, a collective name representing a family of de-N-acetylated chitins deacetylated to different degrees. Generically, the term chitosan has been applied when the extent of deacetylation is above 70% and the term chitin is used when the extent of deacetylation is insignificant, or below 20% (102).Chitosan polymers may present different molecular weights (MW) (50–2000 kDa), viscosity and degree of deacetylation (DD) (40–98%).

Recently, the commercial value of chitin has increased due to the beneficial properties associated with its soluble derivatives, applied essentially in the fields of chemistry, biotechnology, agriculture, food processing, medicine, dentistry, veterinary, environmental protection and paper or textile production. Both chitin and chitosan exhibit valuable biological activities, which have made these polysaccharides increasingly popular. Typical activities include antitumor, anticarcinogenic, immunoadjuvant, hypolipidemic, hemostatic, promotion of wound healing, prebiotic by enhancement of probiotic bacteria growth (e.g. Lactobacillus bifidus) and antimicrobial (103). Besides this, other positive aspects include the fact that they are derived from a natural source, biologically reproducible, biodegradable, biocompatible, non-toxic, biologically functional and changeable in molecular structure.

Chitin and chitosan are structurally similar to heparin, chondroitin sulphate and hyaluronic acid, which are biologically important mucopolysaccharides in all mammals. Chitosan is almost the only cationic polysaccharide in nature (104) rendering unique properties in regard to biomedical applications.

Antimicrobial properties

Chitosan has shown antimicrobial activity against a wide range of target organisms, including Gram-positive and -negative bacteria, yeasts and moulds.

The antimicrobial effect varies considerably with the molecular structure – both degree of polymerization and level of deacetylation affect independently the antimicrobial activity of chitosan, though it has been suggested that the influence of the MW on the antimicrobial activity is greater than the influence of the DD.

Although the information about antibacterial activity of chitosan is still limited, the mostly accepted mechanism of action upon bacteria explains that the physiological pH in the cell is around neutral, which makes chitosan water-insoluble molecules to precipitate, and stack on the microbial cell surface. Therefore, an impermeable layer around the cell is formed, blocking the channels, which are crucial for living cells. The formation of this layer around the cell prevent the transport of essential solutes (causing internal osmotic imbalances) and may also destabilize the cell wall beyond repair, thus inducing severe leakage of intracellular electrolytes such as potassium ions and other low MW constituents (e.g. proteins, nucleic acids, glucose, and lactate dehydrogenase) and ultimately cell death (105).

Another proposed mechanism is the interaction of chitosan oligosaccharides (COS) with bacterial DNA, which leads to the inhibition of the mRNA and protein synthesis, via the integration of COS into the nuclei of the microorganisms (106).This mechanism of action has been reported mainly in Gram-negative bacteria, where the thin layer of peptidoglycan on the cell wall facilitates to get through (107).

A third mechanism proposed for chitosan and COS involves metals chelation, suppression of spore elements and binding to essential nutrients required for microbial growth (108).

Chitosan also exhibits antifungal activity upon moulds and yeasts. This activity is assumed to be fungistatic rather than fungicidal. Generally, chitosan has been reported as being very effective in inhibiting spore germination, germ tube elongation and radial growth. The antifungal mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly upon fungal growth, similarly to the effects observed in bacteria cells. The inhibition mechanism of COS against fungi is also similar to that of bacteria explained above. Microscopic observation showed that COS diffuse inside hyphae interfering on the enzymes activity responsible for the fungus growth. The damaging efficiency of chitosan upon fungal cell walls is also dependant on the concentration, DD and pH of the surrounding environment (109).

Immunomodulatory properties

The strong immune stimulatory activity attributed to chitosan derivatives has been linked to the presence of N-acetyl-D-glucosamine residues (110). High MW chitosan upregulates production of IL-1, TNF-**α**, granulocyte macrophage colony stimulating factor (GM-CSF), nitric oxide (NO) and interleukin-6 (IL-6) in macrophages. Besides this, COS enhances TNF- **α** and IL-1 **β** release by macrophages (111). Chitosan also enhances the functionality of inflammatory cells such as PMN, macrophages and fibroblasts (110) .

Repairing activity

N-acetylglucosamine is the monomeric unit of chitin, but also occurs in hyaluronic acid, an extracellular macromolecule that is implicated in wound repair. Ueno et al. (112) demonstrated that chitosan incorporated in cotton enhanced wound healing by promoting infiltration of PMN cells at the wound site and then inducing active bio debridement by these cells (113), an essential process in wound healing. Chitosan-treated wounds showed histologically regeneration signs such as severe polymorphonuclear leukocyte infiltration with increased granulation, higher amounts of collagen and osteopontin at the wound site (114).

In a study using chitosan dressed skin grafts, Stone and collaborators (115) demonstrated that, as a semi-permeable biological dressing, chitosan maintained a sterile wound exudate beneath a dry scab, preventing dehydration and contamination, thus improving conditions for healing. Furthermore, it facilitated wound re-epithelization and nerve regeneration within a vascular dermis.

2.2.2 Chitosan coated garments

Recently, a garment coated with a medium molecular weight chitosan derived from shrimp shells, with >75% deacetylation, was developed. Its antibacterial activity had been proven (116) and the skin tolerability of this textile was ascertained in healthy individuals; its comfort was evaluated in a small number of AD with severe AD (data not published from the 2nd Dermis Project).

The 2nd Dermis project was developed between 2008 and 2011, it was promoted by the Portuguese textile enterprise Crispim Abreu, Lda with financial support from National strategic Plan Portaria 1462/2007 “15th November on behalf of incentive to investigation and technological development with the objective of developing a cotton textile coated with chitosan. It involved the participation of various entities: Pharmacy Faculty of Porto University, Biotechnological School of Catholic University, Textile and Clothing Technologic Centre (CITEVE) and Centre of Nanotechnology, Functional and Smart materials (CENTI) .The Immunology Laboratory of Faculty of Medicine of Porto University participated as medical consultant of this project.

3. Aims of the study

The purpose of this study was to investigate the influence of skin microbiologic, genetic, immunoallergic and psychological factors; as well as the efficacy, safety and immune modulator effects of chitosan functional textiles in atopic dermatitis patients.

Specific questions for the study programme were:

1. Is the staphylococcal community profile of atopic dermatitis patients different from healthy subjects from a molecular point of view (**Study I**)? Is there any relation between Fillaggrin p.Arg501Ter, 2282del4 mutations, Pro478Ser SNP and colonization with Staphylococci species, immunoallergic markers and disease severity (**Study II**)?
2. Can personality traits and psychological distress impact on disease severity and quality of life of patients with long term atopic dermatitis (**Study III)?**
3. What are the evidence based clinical recommendations on the use of functional textiles in the management of atopic dermatitis (**Study IV**)?
4. What is the efficacy and safety of chitosan coated garments in the management of atopic dermatitis (**Study V**)?
5. What are the immunoallergic effects of the use of chitosan coated garments in atopic dermatitis patients? (**Study VI**)?

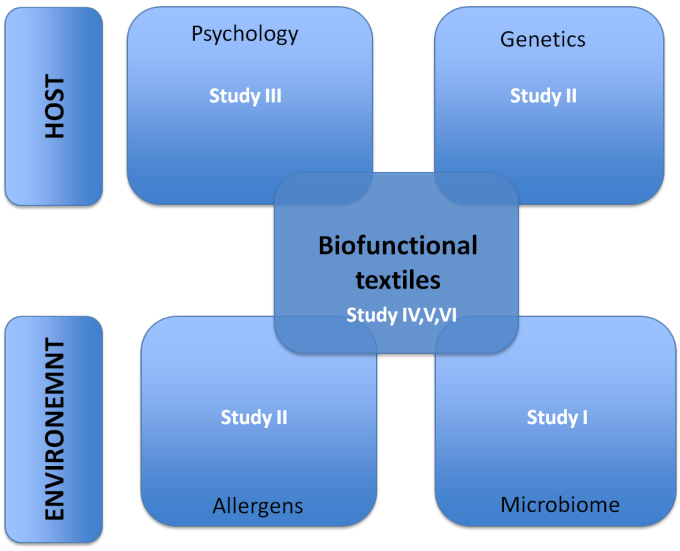

Figure 1.a *Framework of studies addressing the AD model*

4. Materials and Methods

4.1 Participants and study design

This thesis is based on three types of studies:

1. Cross sectional analysis of patients with AD assessing the relation between the Staphylococci skin profile, genetic and immunoallergic factors (Study **I**, **II**) and impact of psychological factors in disease severity (Study **III**).
2. A systematic literature review of studies, and their meta-analysis, that have used functional textiles to manage AD (Study **IV**).
3. Randomized controlled trials assessing the efficacy and safety of chitosan coated garments (Study **V**), and its immunomodulator effects (Study **VI**).

A total of 78 AD patients participated in the randomized controlled trials, and 87 patients and 24 healthy controls in the cross sectional surveys. A summary of the study subjects and design is shown in **Table 2**.

Table 2 *Summary of subjects and study design*

Study	Design and subjects	Gender (f/m)	Age,y (SD)	Atopic (%)	Participants	Intervention	Duration
I	Cross sectional, n=33 (9 patients, 24 controls)	15/19	28 (8)	na	AD patients followed in Allergy setting, healthy volunteers	na	na
II	Cross sectional, n=73	45/29	30 (13)	77	Adolescents and adults patients with AD	na	na
III	Cross sectional, n=44	27/17	31 (13)	73	Adolescents and adults patients with AD	na	na
IV	Systematic review with meta-analysis	na	na	na	na	na	na
V	Randomized double blind placebo controlled, n=78	44/34	31 (13)	64	Adolescents and adults patients with AD	Chitosan coated garments	2 months
VI	Randomized double blind placebo controlled, n=78	44/34	31 (13)	64	Adolescents and adults patients with AD	Chitosan coated garments	2 months

*SD-standard deviation, na-not applicable

4.1.1 Diversity profile from the staphylococcal community on atopic dermatitis skin: molecular approach (Study I)

Consecutive patients older than 2 years old attending an allergy clinic with medical diagnosis of AD according to the criteria of Hanifin and Rajka and clinically apparent AD lesions without signs of secondary infection were invited to participate. Patients taking systemic antibiotics; topical corticosteroids, calcineurin inhibitors or immunosuppressants during the previous 2 weeks of medical

observation and sampling were excluded. Twenty-four healthy subjects were used as controls.

Study protocol included assessment of AD severity by SCORAD index, identification of bacteria belonging to the Staphylococcus genus, both S. aureus and coagulase-negative Staphylococcus (*S. capitis, S. epidermidis, S. haemolyticus and S. Hominis*), and identification of staphylococci genes that encoded staphylococcal enterotoxins (SEs), SE-like toxins and toxic shock syndrome toxin-1.

4.1.2 Relation between FLG genetic profile, skin colonization with *S.aureus*, immunoallergic markers and disease severity (Study II)

Consecutive patients older than 12 years, diagnosed with AD according to the criteria of Hanifin and Rajka (69) that were being recruited to a randomized clinical trial (see detailed description on Study **IV,V**) were invited to participate. Participants with severe skin disease other than AD, such as contact, seborrheic dermatitis, nummular eczema, hand eczema, psoriasis; secondary infection with bacteria, fungi, or virus; any major systemic disease that could interfere with study procedures or assessments were excluded.

Study protocol included assessment of identification of FLG gene mutations Mp.Arg501Ter, c.2282del4 and p.Pro478Ser polymorphism, skin microbiological characterization (total staphylococci and *S.aureus* number of colony forming units), determination of inflammatory and allergic systemic serum markers as total IgE, eosinophil cationic protein (ECP) and specific IgE to a mixture of inhalant allergens (Phadiatop™), *S. aureus* enterotoxins A, B, C, TSST and *Malassezia spp* (ImmunoCap ™), AD severity by SCORAD index. Information on previous medical diagnosis of asthma and previous medication was obtained by interview.

4.1.3. Psychological factors and AD (Study III)

Consecutive patients, older than 18 years old, with medical diagnosis of AD, were invited to participate. Exclusion criteria were other skin immunemediated skin diseases such as seborrheic dermatitis, nummular eczema, hand eczema, psoriasis; any major systemic disease that could interfere with study assessments were excluded.

Study protocol included assessment of personality traits, anxiety and depression levels, Dermatological quality of life by questionnaire and AD severity trough medical evaluation with a Score of severity of atopic dermatitis (SCORAD index). Information on disease duration, atopy, and previous medical diagnosis of asthma was obtained by interview.

4.1.4 Functional textiles and AD (Study IV)

A detailed description of the systematic review procedures is presented in the original article and its addenda.

We selected published reports of randomized controlled trials (RCTs), observational and case studies (with a cohort or case-control design) that compared or assessed the effects of functional textiles in

patients of any age with a clinical diagnosis of atopic dermatitis; no restrictions were placed on disease severity or previous or current treatment.

The primary outcome was defined as changes in overall AD severity, measured by the SCORAD index and other scales for evaluating AD severity (21). Secondary outcomes included changes in symptoms, quality of life, need for rescue medication, microbiological skin flora composition, epidermal skin physiology and safety.

Electronic searches were undertaken in 3 large biomedical databases: the Cochrane Central Register of Controlled Trials, Scopus, and Medline. We used the following keywords (first group): “atopic eczema dermatitis syndrome”, ‘atopic dermatitis’, ‘atopic eczema’, coupled with (second group) ‘textiles’, ‘fabrics’, ‘garments’, ‘clothes’, ‘dressings’. *A priori* inclusion criteria limited retrieved articles to those assessing the use of textiles in individuals with AD. Subsequently, each study was evaluated to determine whether it met the entry criteria for the review. Hand searches of the reference lists of all pertinent reviews were performed and potentially relevant studies identified. Abstracts from relevant conferences were also searched. After the electronic literature searches, using the title, abstract or both, two authors independently selected articles for full-text scrutiny. The authors agreed on a set of articles, which were retrieved and assessed to determine compliance with the entry criteria. Information regarding the following characteristics was extracted from each study: design (description of randomization, blinding, number of study centres, and number of study withdrawals); participants (sample size), mean age, age range of the population; intervention (type and study duration); and outcomes (type of analysis and outcomes analysed). The results of comparable studies for a specific outcome were pooled using a random effects meta-analysis (117).

Grading system- evidence was graded based on an analysis of outcome measures. The overall quality of evidence is presented using the GRADE approach recommended by the Cochrane Handbook for Systematic Reviews of Interventions (117). That is, for each specific outcome, five factors were scrutinized: (1) limitations of the study design or the potential for bias across all studies accordingly to the measure of a particular outcome, (2) consistency of results, (3) directness (generalizability), (4) precision (sufficient data), and (5) the potential for publication bias. The overall quality was considered to be high if multiple RCTs with a low risk of bias provided consistent, generalizable results for the outcome. The quality of evidence was downgraded by one level if one of the factors described above was not met. Likewise, if two or three factors were not met, two or three levels downgraded the level of evidence, respectively. Thus, the GRADE approach resulted in four levels of quality of evidence: high, moderate, low, and very low. When a given outcome was measured by only one study, data were considered to be “sparse’, and subsequently the evidence was labelled as “low quality. The systematic approach suggested by the GRADE working group was followed using the GRADE profiler software (version 3.2) (118-122).

Quality of evidence classification was needed to ascertain if an estimate of the effect is adequate to support a particular recommendation for the clinician. Strength of recommendation was performed according to the quality of the supporting evidence and classified as strong or weak for the use of

functional textiles, through the balance of desirable/undesirable outcomes (118-122).

4.1.5 Efficacy and safety of chitosan coated garments (Study V and VI)

Studies **V** and **VI** are a randomized, double-blind, placebo-controlled, single-center trial. **Figure 2** shows the flow of participants. Ethics committee approved the study at 6th September 2011, patients recruitment and follow up occurred between December 2011 and June 2012.

Subjects were invited to participate in the trial during hospital visits, through trial posters on bulletin boards in hospitals, newspaper and Internet advertisements.

Subjects older than 12 years with a diagnosis of AD (69) were eligible for participation following provision of written informed consent. Patients with severe skin disease other than AD (e.g., psoriasis); secondary infections; major systemic diseases; women who were pregnant and subjects unable to comply with study and follow-up procedures were excluded

Patients who met any of the following criteria were withdrawn from the study: use of topical or systemic antibiotics during the study; withdrawal of consent; detection of significant protocol violations and investigator's decision to withdraw the patient due to adverse effects such as skin infections.

Subjects were randomly assigned to one of two interventions through computer-generated random numbers. The randomization was performed by an independent researcher;the randomization table and intervention codes were kept by the independent researcher in an opaque sealed envelope up to completion of data analysis. A study nurse established phone contact with the independent researcher, who informed the nurse which treatment package was to be assigned to which patient.

A hundred and two patients were assessed for eligibility; 24 were excluded because they did not meet inclusion criteria; 22 because the medical diagnosis of AD was not confirmed by the investigation team; and two because of significant comorbidities (multiple sclerosis and diabetes mellitus type 1). 78 were randomized; 35 to placebo and 43 to chitosan groups. In the chitosan group, two patients were lost before receiving the intervention and one patient decided to withdraw because of disease progression. In both groups, three patients were lost to follow up due to their inability to keep their scheduled medical visits (**Figure 2**).

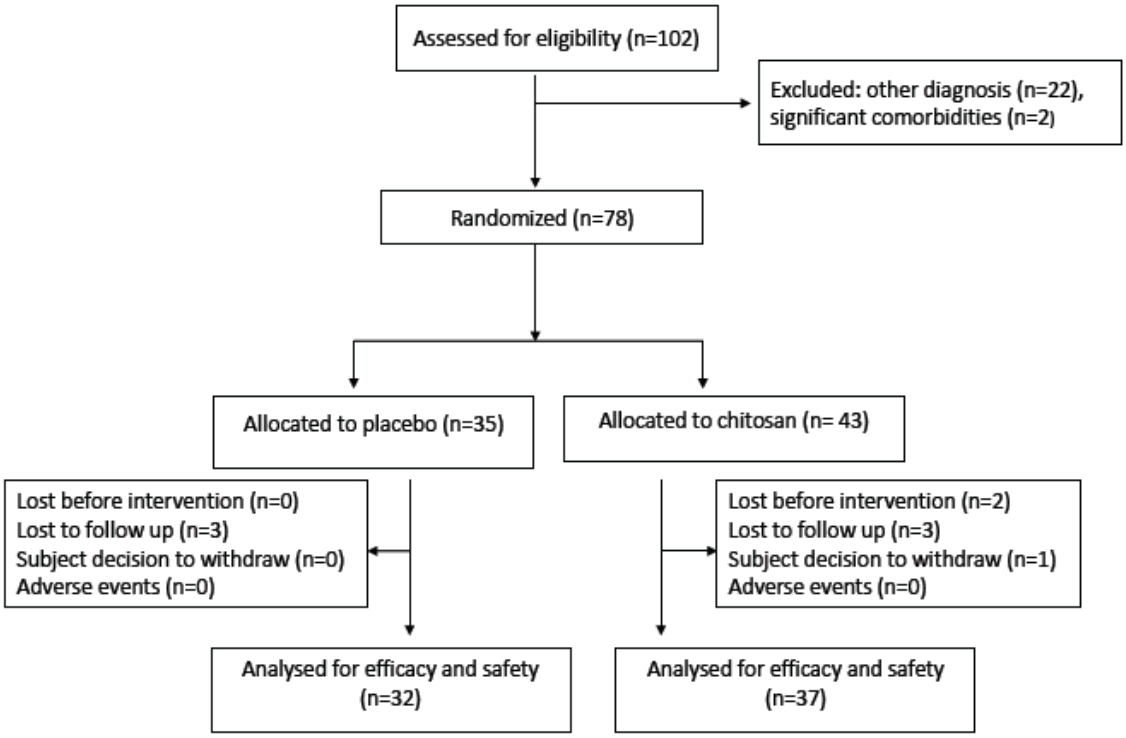

Figure 2 *Flow chart for Study V and VI*

The study consisted of a 2-week run-in period and an intervention period of 8 weeks (**Table 3**). Eligibility to participate was determined at the screening visit. At the end of the run-in period, the patients were examined by the same physician as in the first visit and those with a change in SCORAD of below 10% with respect to baseline were considered eligible for randomization. Participants were randomized to receive either an uncoated pair of cotton pyjamas or a pair of cotton pyjamas coated with chitosan (ChitoClear CG-800). The pyjamas, placed in a sealed plastic package, consisted of a long-sleeved top and long pants to be worn at night for the duration of the study. Both pyjamas were made of 100% organic cotton, without dyes or preservatives, and were visually indistinguishable from each other. The *in vitro* antibacterial activity of the chitosan-coated textile was shown to persist after 30 washing cycles (123) and washing durability was studied through washing assays at 40ºC (123).

Table 3 *Study plan of Study V*

	Screening (W -2)	Baseline (D 0)	Daily registries W 0 to 8	Final (W 8)
Informed consent	√			
Demographic characteristics	√			
Inclusion/exclusion criteria check	√	√	√	√
Randomization		√		
SCORAD index	√	√		√
Dermatology Life Quality Index		√		√
Skin Microbiological characterization		√		√
Pruritus daily score		√	√	
Sleep loss daily score		√	√	
Rescue medication			√	
Current medication	√		√	
Adverse event			√	√

D-Day; W-week

4.1.6 Immunomodulator effects of chitosan coated garments (Study VI)

Participants and study design were similar to **Study V**.

Study protocol included the assessment of immunoallergic serum markers before and after the intervention. See flow chart for **Study V** and **VI** in **Figure 2**.

4.2 Measurements

A summary of the study outcomes and instruments is shown in **Table**

Table 4 *Summary of the study outcomes and instrument*

Outcomes	Instruments	Study					Ref
		I	II	III	V	VI	
Clinical assessment							
Severity	SCORAD index	x	x	x	x	x	(69)
Symptoms	Diary				x		na
Rescue medication	Diary				x		na
Flares	Diary				x		na
Control	Diary				x		(124)
Quality of life	Dermatology life quality index			x	x		(125)
Safety					x		na
Psychological assessment							
Anxiety	Hospital Anxiety and Depression Scale			x			(126)
Depression	Hospital Anxiety and Depression Scale			x			(126)
Personality	NEO Personality Inventory (NEO-PI-R)			x			(127)
Skin Microbiome							
Total Staphylocci counts		x	x		x		na
S.aureus counts		x	x		x		na
Molecular identification	Multiplex PCR	x					(128)
Allergic and inflammatory systemic markers							
IgE	CAP system (Phadia, Sweden)					x	mi
Specific IgE	CAP system (Phadia, Sweden)					x	mi
Phadiatop® test	CAP system (Phadia, Sweden)					x	mi
Eosinophil cationic protein	CAP system (Phadia, Sweden)					x	mi
Genetic characterization							
Filaggrin mutations	amplification of exon 3 FLG gene and direct sequentiation by Sanger method		x				mi
Filaggrin polymorphisms	amplification of exon 3 FLG gene and direct sequentiation by Sanger method		x				mi

mi: according with manufacturer instructions; na: not aplicable

4.2.1 Skin Microbiological profile: molecular approach (Study I), standard cultural methods (Study II, V)

In **Study I,** the sampling procedure was performed on 25 cm^2 of the skin area on popliteal and/or antecubital crease from patients and controls, in **Study II and V,** in the same area, but on popliteal and antecubital crease bilaterally and interscapular region. The skin within the enclosed area was scrubbed using a sterile swab moistened with dilution liquid. The tip of the swab was then broken against the wall of a glass tube containing the dilution liquid, and the tube was immediately capped and shaken to suspend the bacteria. The samples were cultured in Baird Parker medium (BPM; Lab M, Lancashire, UK) by spread plate technique in duplicate and incubated at 37°C during 24–48 h.

In **Study I,** the DNA was isolated as previously described (128). The type reference strains used were as follows: *S. aureus* ATCC 25923, *S. epidermidis* ATCC 14990, *S. capitis* ATCC 27840, *S. haemolyticus* ATCC 29970 and *S. hominis subsp. hominis* ATCC 27844. In addition, the type strains of *S. aureus* that are described in Soares et al. (128) were used as positive controls for Sag genes. Regarding molecular identification of the staphylococcal isolates, the wild isolates obtained were submitted to multiplex PCR with the primers FemF/FemR, SepF/SepR, ScapF/ScapR, ShaemF/ShaemR and ShomF/ShomR for the identification of the *S. aureus, S. epidermidis, S. capitis, S. haemolyticus* and *S. hominis* species, respectively.

Complementary identification of three (or one in the case of *S. lentus*) isolates for each species formerly identified by multiplex PCR was performed by 40oda gene sequencing.

21 of the 69 isolates previously identified as *S.aureus* strains were screened for Sag gene detection. They were randomly selected and consist of five to six isolates per individual positive for *S. aureus*. The primers were first applied individually, and multiplex PCR conditions were prepared.For virulence factor detection, namely coagulase production, Dnase and haemolytic activity, strains were tested as demonstrated by Soares et al. (128)

In **Study V,** samples were kept refrigerated at 4º C after collection and transported to the laboratory. They were decimally diluted and plated in Baird-Parker agar (BPA) and Mannitol Salt agar (MSA) using the spread plate technique (by inoculating with 25mL of the diluted sample) within 2 hours after sampling. Following 48h incubation at 37ºC, CFU per mL were determined upon enumeration of colonies on general (PCA, for total aerobic counts) and selective/differential media (MSA for total staphylococci and BPA for *S. aureus*), respectively. Microbiological outcome measures were mean changes in colony forming units (CFUs) per 100 cm^2 of total staphylococci (*S. aureus* plus coagulase negative staphylococcus species) and *S. aureus* isolates.

4.2.2 Psychological assessment (Study III)

Personality traits were assessed through the short version of the NEO Personality Inventory (NEO-PI-R) (129). This 60-item multiple-choice questionnaire evaluates the 5 main dimensions of personality: Neuroticism (as a measure for emotional stability or lability), Openness (as the predisposition to new experiences), Extraversion (as the main energy focus being held in- or outwards), Agreeableness (as the ability to deal with others) and Conscientiousness (as the sense of right and wrong towards own behaviour). NEO-PI-R has already been validated to the Portuguese population (130).

Anxiety and Depression were evaluated through the Portuguese version of the *Hospital Anxiety and Depression Scale* (HADS) (131). This test has two separate scales: one for anxiety (HADS-A) and one for depression (HADS-D). Both sub-scales are graded from 0 (best) to 21 (worst) and then divided into Normal (if score ≤ 7), mild (8 to 10), moderate (11 to 14) and severe (if ≥ 15). This score has already been validated to the Portuguese population (126).

Quality of Life (QoL) was assessed by the *Dermatology Life Quality Index* (132), validated in the Portuguese population; a 10-item questionnaire for patients above 16 years, aiming at evaluating the

patients' perception of the impact of the skin diseases on several aspects of QoL, over the past week. Scores range from 0 (no effect) to 30 (severe impairment on QoL). Patients scoring 0 to 1 were categorised as having no impact on QoL, 2 to 5 as having a mild impact, 6 to 10 as a moderate impact, 11 to 20 as a severe impact and patients scoring 21 to 30 as having an extremely severe impact on QoL.

4.2.3 Clinical assessment (Study V)

The primary efficacy outcome measure was mean relative and absolute change in disease severity after the intervention assessed by SCORAD (69). The total possible score ranges from 0 to 103.

Secondary outcome measures were number of patients with a minimal clinically important difference in SCORAD post-intervention; mean change in quality of life score; changes in daily pruritus and sleep loss scores; need for rescue medication; number of flares; number of totally controlled weeks (TCWs) and well-controlled weeks (WCWs); and number and severity of adverse events during the 8-week study period.

Patients were characterized according to age, gender, current medication, personal history of atopy, self-reported medical diagnosis of asthma, disease duration and disease severity. The SCORAD index was used to classify AD as mild (score ≤15), moderate (16-39), or severe (>40) (133). During the baseline and final visits, participants were asked to complete the Portuguese version of the Dermatology Life Quality Index (DLQI) or, if they were younger than 16 years, the children´s version of the questionnaire.

Participants recorded and scored daily symptoms of pruritus and sleep loss according to the 10-point VAS, and registered all medication use during the study period. Rescue medication was defined as any treatment, other than emollient, applied in response to disease worsening (i.e. escalation of treatment). A flare was defined as an episode requiring rescue medication for 3 or more consecutive days; a TCW as a a seven-day period without need of rescue treatment and without any days of sleep loss or pruritus score above a pruritus score of above 4; and a WCW as a 7-day period with need for rescue treatment or with a sleep loss or pruritus score of above 4 for no more than 2 days (134).

Patients were asked to report any adverse reactions that could have occurred during the 8 weeks study period to the research team. Adverse events that were transient and easily tolerated by the patient were considered mild, moderate if causing discomfort and interrupting the subject's usual activities, severe if the event caused considerable interference with the subject's usual activities and could be incapacitating or life-threatening. The principal investigator defined adverse events as not, possible, probably, or definitely related to treatment.

4.2.4 Immunoallergic systemic assessment (Study II, VI)

Total serum IgE concentrations were determined with ImmunoCap FEIA (fluorescence enzyme

immunoassay) test system (Thermo Fisher Scientific, Phadia®). Values <2.00 kU_A/L were defined as absent or undetectable total serum IgE. Specific IgE sensitization for inhalant allergens was tested with the Phadiatop® containing a mixture of common environmental allergens (values < 0.10 PAU/L were defined as absent or undetectable). Specific IgE to *S. aureus* enterotoxins A, B, C and TSST and to *Malassezia spp* was detected by ImmunoCAP FEIA assay using ImmunoCAP250 (Thermo Fisher Scientific, Phadia®) following the manufacturer´s instructions. Values < 0.10 kU_A/L were defined as absent or undetectable. Results ≥0.35 kU_A/L were considered positive.

Eosinophil cationic protein (ECP) was measured by fluorometric enzyme immunoassay (FEIA) (Phadia, Uppsala, Sweden). Values over 15 µg/L were considered elevated. Blood samples were collected by venipuncture using Terumo Venosafe® Serum-Gel tubes. Serum was separated within 30 min of blood collection, after centrifugation 10 min at 400x g. Aliquots of serum were stored at -80ºC until use.

4.3 Statistical analysis

The data analysis was performed with SPSS software, version 20.0.

In **Study II, III, and V**, the power calculation was made based on SCORAD minimal clinically important difference in change. A total of 42 patients were needed in a two-treatment parallel-design study to detect a treatment difference at a two-sided 0.05 significance level with a probability of 81 percent if the true difference in SCORAD between treatments was 8.7 units (based on the assumption that the standard deviation of the response variable was 9.6) (135). Probiotics were considered to have a similar effect power on intervention and in **Study VI,** sample size calculations were based on a previous randomized clinical trial with probiotics assessing significant changes in ECP serum levels in AD patients (136). A total of 62 patients were need in a two-treatment parallel-design study to detect a treatment difference at a two-sided 0.05 significance level with a probability of 81 percent if the true difference between treatments was 22.0 units (based on the assumption that the standard deviation of the response variable was 30.0).

In **Study III**, personality traits were recoded into a 3-item category with patients scoring ‘low’ or ‘very low’ grouped into ‘Low’ and patients scoring ‘high’ or ‘very high’ grouped into ‘High’.

Comparison of categorical variables with continuous variables presenting a normal distribution was performed by one-way ANOVA test; when significant differences were found a post-Hoc Bonferroni correction was performed. Correlation between continuous variables was achieved through the Spearman correlation coefficient (**Study III**). For other associations (**Study II, V** and **VI**) Wilcoxon ranked sign test was used for nonparametric analysis of related groups and Mann-Whitney for independent samples.

In the systematic review analysis (**Study IV**), when data was available for a pooled estimate of the impact of intervention, it was intended that meta-analyses would be conducted for direct comparisons. Effect measures were presented with 95% confidence intervals. We present weighted mean differences (WMD) and 95% confidence intervals for continuous outcomes for each randomized

controlled trial. In order to determine whether combining the results was appropriate, a heterogeneity test (Chi² statistic) was performed. We used a fixed-effects model to estimate the pooled effect when no evidence of heterogeneity was detected (Whitehead and Whitehead, 1991). However, if evidence of heterogeneity was observed, we used a random-effects model (DerSimonian and Laird, 1986). Analysis and forest plots were produced using RevMan 5 Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012.

The analysis of the randomized controlled studies (**Study V** and **VI**) was conducted using the "intention-to-treat" (ITT) approach, including all randomized subjects. In **Study V**, missing data for the patient who withdrew from the study before its completion were estimated using an expectation-maximization algorithm. Overall, data referring to symptoms diary was imputed in one patient from active group. Because of their skewed distributions, comparisons of SCORAD were made after logarithmic transformation. Whenever necessary to permit analysis in the log scale, a constant (0.1) was added to each value to eliminate 0 values. Changes within groups were compared using paired t-test and and differences between groups were compared by analysis of covariance (ANOVA) with baseline value as covariate. When significant differences were found in one-way ANOVA test, a post-Hoc Bonferroni correction was performed. For study **V**, the interaction term between time and intervention was used to compare time trends between groups in several outcomes (number of days per week with need of rescue medication, sleep loss and pruritus symptoms). Mixed effects models with random intercept and time slope by individual were used to estimate the interaction term.

4.4. Ethics

Studies protocols were approved by the Ethics Committee of Porto University, (study **I**, **II**, **III V** and **VI**). The subjects themselves (study **I, II**, **III, V, VI**), or their parents (study **I**, **II, V, VI**) provided their written informed consent.

5. Results

5.1. Participants

The characteristic of the subjects participating in studies **I**, **II**, **III**, **V** and **VI** are presented in **Table 5**.

Table 5 *Summary of participants characteristics*

Studies	I		II	III	V, VI	
	AD	Healthy	AD	AD	AD	
					Placebo	Chitosan
n	9	24	73	44	35	43
Age, yr (range)	15 (3-35)	22 (19-33)	30 (13-68)	31 (16-53)	27 (12-85)	26 (12-65)
Sex (f:m)	4:5	11:13	44:30	27:17	21:14	23:20
Disease duration,y	7 (6)	na	16 (10)	17 (10)	15(9)	17 (11)
SCORAD (0-103)	44 (6)	na	42 (24)	46 (28)	44 (27)	42 (21)
Atopic, n (%)	8(89)	na	50(68)	32(73)	21 (60)	29 (70)
Asthmatic n,(%)	6 (67)	0	39(53)	27(61)	18 (51)	21 (49)
QoL(0-20)	nd	nd	nd	8.6 (5)	8 (5)	8 (4)

Data presented as mean (SD) unless otherwise indicated; *atopy defined by the result of Phadiatop®; SCORAD: score index of AD severity QoL; quality of life nd: not done; na: not applicable. In studies IV and V baseline groups characteristics were compared using Chi-Square or t-test where appropriate and no differences were observed

In **Study I**, nine patients (aged 3–35 years) with moderate to severe AD and 24 healthy controls (aged 19-33) were included.

In **Study II**, data from 73 patients (30±13 years, 61% female, 77% atopic) with AD for 16±10 years were analyzed.

In **Study III**, anxiety was present in about a third (n=15) of the patients, mostly mild (n=9). Only 6 (14%) of patients presented depression (five mild, one moderate). As for personality traits, most patients scored normal on all five dimensions and most patients reported a moderate impact of AD in their life's quality.

In **Study V** and **VI,** no major imbalances were found in the baseline characteristics of the individuals included in the placebo and chitosan groups: most patients were adult, with AD for more than 10 years, more than half were female, and the majority were atopic and had self reported previous history of asthma (**Table 5**). Oral antihistamines and topical steroids were used by most patients, almost half had been prescribed at least once oral steroids in the last year and a systemic immunosupressor such as cyclopsorin in 17% overall. Similar proportion of participants with mild (2 versus 5), moderate (19 versus 14) and severe (22 versus 16) AD occurred respectively in chitosan and placebo intervened groups.

5.2. Diversity profile from the staphylococcal community on atopic dermatitis skin: molecular approach (Study I)

Staphylococcus identification

Six of the nine skin samples from AD patients were positive for Staphylococcus spp. From the six individuals positive for Staphylococcus spp., as determined by the BPM counts, the staphylococcal microflora was dominated by *S.aureus* (69 isolates, 35.6%) followed by *S. epidermidis* (59 isolates, 30.4%) and *S. hominis* (54 isolates, 27.8%) species.

The samples from healthy individuals were characterized by a greater heterogeneity in terms of the number of identified species, viz. *S.aureus* (eight isolates), *S. capitis* (four isolates), *S. epidermidis* (four isolates), *S. haemolyticus* (five isolates) and *S. hominis* (four isolates), *S. lentus* (one isolate), *S. lugdunensis* (three isolates), *S. saprophyticus* (nine isolates) and *S. warneri* (10 isolates) as seen in **Figure 3**.

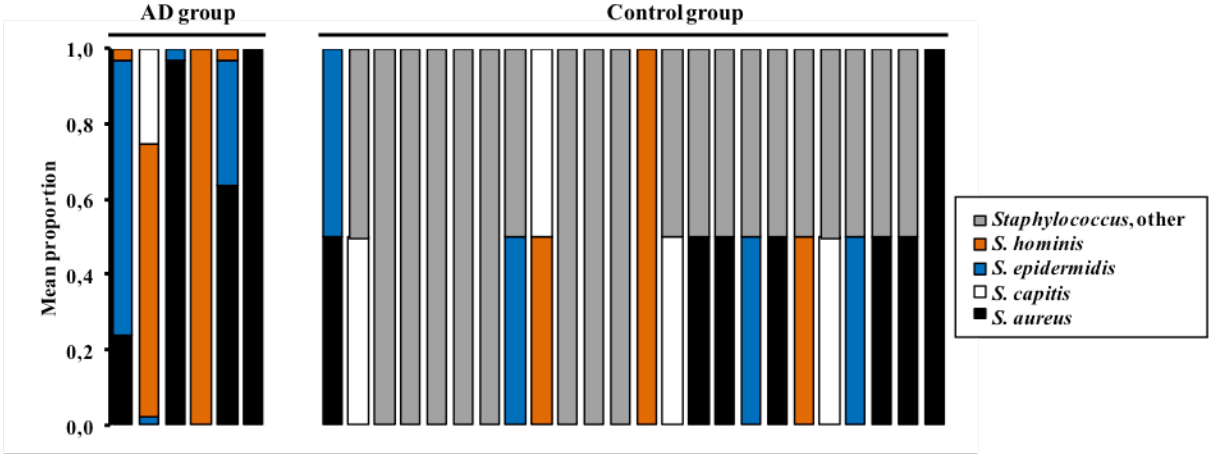

Figure 3 *Diversity and bacterial taxonomic classifications with mean relative abundance of the staphylococcal species identified from the skin of controls and AD patients.*

SuperAntigenes detection

Twenty one *S.aureus* strains isolated from AD skin were tested, and 16 (76%) of them were SAg-positive strains (**Figure 4**). The most frequently detected genes were for enterotoxins SEG, SEIM, SEIN and SEIO (15 isolates, 71%), and they were always found together in the same isolate, with the exception for one isolate where we did not detect the SEIO gene. Enterotoxin A (six isolates, 29%) and SEIL (seven isolates, 33%) were also frequently detected along with the SEG, SEIM, SEIN and SEIO genes. The classical SEs (SEA-SEE) were not detected in our samples. The SEIU gene (one isolate, 5%), which was rarely detected, was also found together with the SEG, SEIM, SEIN and SEIO genes.

Sixty nine (35.6%) and eight (16.7%) isolates among AD and control individuals, respectively, were

positive for both the presence of coagulase and DNase and correlated with the *S.aureus* isolates as expected. In terms of haemolytic activity as performed in horse blood agar, a similar occurrence was seen in control (39.6%) and AD individuals (33.7%).

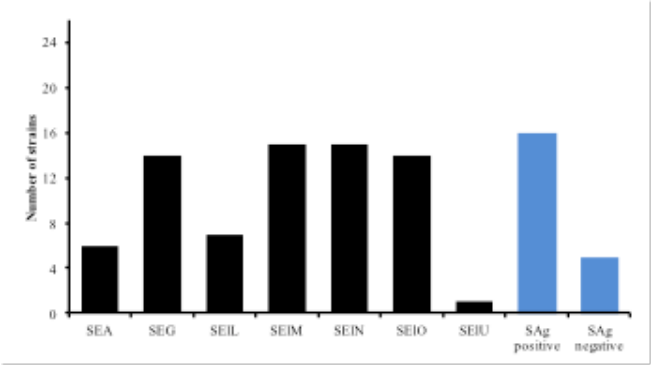

Figure 4 *Diversity of SAg genes detected and prevalence of toxigenic and nontoxigenic strains of* S. aureus *isolated from the skin of Portuguese patients with AD and of healthy control subjects*

5.3 Relation between FLG genetic profile, skin colonization with *S.aureus*, immunoallergic markers and disease severity (Study II)

FLG mutations were present in 15% of patients (9 p.Arg501Ter and 2 c.2282del4), p.Pro478Ser in 38% of cases (3 homozygotes, 25 heterozygotes); p.Pro478Ser was in linkage disequilibrium with the null-mutations and three patients with p.Arg501Ter mutation also had p.Pro478Ser. The presence of p.Pro478Ser was associated with a more severe disease reflected by a higher SCORAD level and severity class as well as increased use of oral steroids (**Table 6**). Furthermore, a significantly higher colonization of *S.aureus* on three of the five sampled regions and higher value of IgE to *S.aureus* Enterotoxin A was observed. Homozygotia for p.Pro478Ser was not an additional risk factor in this particular group of patients. There were no differences between patients with and without *FLG* null-mutations concerning AD severity, inflammatory allergic markers and colonization with *S.aureus*.

Table 6 *Characteristics of atopic dermatitis patients according to the filaggrin genotype*

	FLG-null mutations Mp.Arg501Ter or C.2282del4			FLG polymorphism Pro478Ser		
	Yes, n=11	No, n=62	p	Yes, n=28	No, n=45	p
Age, years	32 (6.1)	29.6 (1.5)	0.91*	34.1 (2.7)	27.3 (1.8)	**0.03***
Female sex, n (%)	7 (63.6)	38 (61.5)	0.22#	16 (57.1)	28 (62.2)	0.42#
Disease duration, years	15.9(10.5)	16.3 (10.4)	0,23*	18.4 (2.3)	14.8(1.3)	0.32*
SCORAD (0-103)	50.2 (30.9)	41.3 (22.6)	0.72*	51.8 (4.2)	36.0(3.4)	**<0.01***
SCORAD severity, n (%)						
Mild	2 (18.2)	5 (8.1)		2 (7.1)	5 (11.1)	
Moderate	3 (27.3)	26 (41.9)	0.81#	6 (21.4)	23 (51.1)	**0.02#**
Severe	6 (54.5)	31 (50.0)		20 (71.4)	17 (37.8)	
Oral steroids, n (%)	3 (27.3)	30 (48.4)	0.22#	17 (60.7)	16 (35.6)	**0.03#**
Atopic, n (%)	6 (54.5)	50 (79)	0.53#	22 (78.6)	34 (75.6)	0.52#
Asthmatic, n (%)	4 (36.4)	36 (58.1)	0.64#	14 (50.0)	26 (57.8)	0.31#
Total IgE, UI/ml	2185 (3294)	4183 (8292)	0.08	6520 (10221)	2240(5228)	0.08*
Phadiatop™, KuA/L	248.6(361)	529.9 (1011)	0.12	763 (1155)	315 (753)	0.13*
ECP	20.7 (14.9)	35.2 (29.1)	0.56	37.2(34.2)	30.5 (21.1)	0.52*
Specific IgE, Kua/L						
Enterotoxin A	0.37 (0.22)	2.4 (1.3)	0.79	4.5 (13.9)	0.46 (0.9)	**0.05***
Enterotoxin B	0.6 (0.26)	1.5 (0.49)	0.42	2.4 (5.1)	0.59 (1.3)	0.23*
Enterotoxin C	1.3 (0.5)	2.2 (0.5)	0.38	2.7 (3.5)	1.56 (3.1)	0.06*
Enterotoxin TSST	0.5 (0.23)	1.4 (0.6)	0.52	2.4 (6.7)	0.42 (0.8)	0.08*
Malassezia	6.2 (5.8)	4.2 (1.1)	0.78	7.2 (13.4)	3.3 (8.7)	0.23*
S.aureus CFU/cm2						
Rigth arm	9471.1	78 152.7	0.48	178 083.3	8002.3	**0.01***
Left arm	158 909.9	70 271.9	0.58	142 859.2	48 310.3	0.92*
Rigth leg	23 454.4	39 728.2	0.91	89 778.9	8 386.7	**0.04***
Left leg	162 754.4	359 865.8	0.96	759 552.7	95 528.5	**0.02***
Neck	8 994.9	30 732.6	0.74	48 538.3	16 244.8	0.80*

ECP: eosinophil cationic protein; SCORAD-scoring atopic dermatitis; CFU-colony forming unit. Results are presented as mean (SD) unless stated otherwise; *Mann Whitney test # Fisher's Exact Test; significant differences presented in bold

5.4 Psychological factors and AD (Study III)

Subjects scoring "high" on Conscientiousness had less severe disease than those scoring 'normal': mean (95% CI) SCORAD of 31.17 (19.58 to 42.58) vs. 56.16 (42.73 to 68.67); p =.039, respectively. No further differences were observed concerning Neuroticism (p =.960), Extroversion (p =.065),Openness (p =.722) or Agreeableness (p =.186) traits (**Table 7**). Depression was weakly, but significantly, correlated with the severity of disease (r_s=.300, p = .046) while no correlations were observed for anxiety (r_s =0.151, p=0.174).

No significant differences were observed between personality traits and the dermatologic life quality index (**Table 7**). Severity of the disease assessed by SCORAD was the main determinant for quality of life (r_s of 0.185; p = .002).

Table 7 *Personality impact on severity and quality of life of atopic dermatitis*

		SCORAD			DLQI		
Personality traits	Categories	Mean	95% CI	p-value*	Mean	95% CI	p-value*
	Low	47.2	22.2 - 71.7		6.7	3.6 - 9.8	
Neuroticism	Normal	45.2	32.5 -57.3	0.960	9.5	7.0 - 11.9	0.235
	High	44.2	28.4 - 58.6		7.9	5.5 - 10.2	
	Low	83.1	-190.7 - 355.7		12.0	-26.1 - 50.1	
Extraversion	Normal	37.1	24.9 - 49.7	0.065	7.4	5.6 - 9.3	0.247
	High	47.2	36.1 - 58.7		8.8	6.5 - 11.1	
	Low	42.2	-10.8 - 95.5		6.7	1.5 - 11.8	
Openness	Normal	48.4	35.0 - 60.6	0.722	8.0	5.9 - 10.0	0.573
	High	41.3	28.5 - 52.8		9.3	6.7 - 11.9	
	Low	55.1	38.1 - 72.5		10.2	7.6 - 12.7	
Agreeableness	Normal	38.3	27.6 - 48.9	0.186	8.0	5.9 - 10.2	0.199
	High	48.1	20.9 - 75.0		6.3	2.5 - 10.1	
	Low	41.1	22.4 - 58.7		8.6	5.9 - 11.2	
Conscientiousness	Normal	56.2	42.7 - 68.7	**0.035**	8.0	5.9 - 10.2	0.885
	High	31.2	19.6 - 42.6		8.8	5.2 - 12.5	

CI: Confidence Interval SCORAD-scoring atopic dermatitis, DLQI-dermatology life quality index.*One-way ANOVA test

5.5 Evidence of efficacy and safety of functional textiles in AD (Study IV)

Thirteen studies met the eligibility criteria and were included in our review. One study, an expert's bibliographic review, was excluded because it did not meet the inclusion criteria (137).

The studies included participants aged between 4 months and 70 years, with no restriction in disease severity. The interventions included silver (89, 90, 93-95), silk (96-101), borage oil (91), and EVOH fiber (92) used for a period of 1 to 12 weeks . RCTs addressed silk textiles in 2 studies (96, 98, 101), silver-coated textiles in 4 (89, 93-95), and borage oil (91) and EVOH fiber (92) in 1 study each. The case-control studies analysed silk fabric (97, 100) and silver-coated textile (90). Silk textiles were also examined in 1 side-by-side comparison study (99) and 1 uncontrolled study (101). Silver-coated fabrics were studied in both children and adults in all cases (89, 90, 93-95). Silk, by contrast, was studied mostly in children (96, 97, 99, 100), and borage oil (11) and EVOH fiber (12) were studied in children only. Control textiles included cotton (for studies of silver, borage oil and EVOH fiber) and regular silk for studies of silk with AEGIS antibacterial treatment (96-98, 100). All the studies addressed eczema severity, measured by SCORAD (89-96, 98, 100) and the Eczema Area and Severity Index (EASI) (99, 101). The skin microbiome was analysed in studies of silver (90, 93, 95) and silk (97), while skin physiology was studied in those of silver and borage oil (91, 94, 95). Safety was assessed in studies of silver (89, 93, 95) and silk (99) textiles. Considering the reported outcomes, all the studies were deemed to have a low or very low quality of evidence (See Table 3, Grade Evidence Profile in published article).

5.5.1 AD severity

SCORAD was used in 10 studies (89-96, 98, 100), involving all kinds of interventions. Compared with placebo, a significant improvement in disease severity was observed for silver in 2 studies (89, 94) and for silk, also in 2 studies (96, 98). In the remaining studies there was a reduction in disease

severity, but no comparisons were made with placebo. Meta-analysis was possible in two RCTs of silver-coated fabrics reporting a reduction in eczema severity (mean difference -12.66 [-21.26; -4.07], I^2>60%) (89, 93) (**Figure 5**).

AD severity (EASI): two studies analysing silk used the EASI to evaluate AD severity. Senti et al., using a side-by-side comparison method, showed a significant decrease in severity, but they did not detect any differences between the side of the body in contact with the treated silk fabric and the other side (99). Kurtz et al. (101), in an uncontrolled study, reported a decrease in EASI following the use of a silk-like bedding fabric.

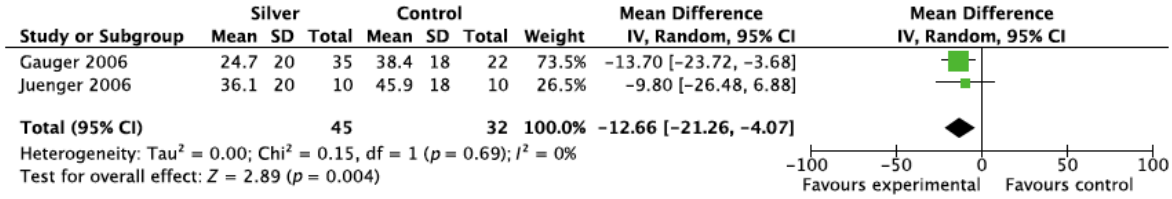

Figure 5 *Meta-analysis of SCORAD results (silver functional textiles versus placebo).*

5.5.2 Symptoms

Five studies, using silver (89, 93), silk (96, 98), and borage oil (91) reported AD symptoms of pruritus and sleep loss as separate outcomes. In the silver group, no significant differences were found in the trial by Gauger et al. (89) for pruritus and sleep loss; Juenger et al. (93), by contrast, showed a significant reduction in symptoms in individuals who used silver textile, but they did not perform a comparison with placebo. In studies examining silk, a significant improvement in symptoms was seen in the active group; these studies were included in a meta-analysis due to their homogeneity (mean difference -1.74 [-2.19; -1.30, I^2 = 0% (96, 98) (**Figure 6**). The trial of cotton undershirts coated with borage oil also reported a reduction in symptoms in the active group, but there was no comparison with placebo (91).

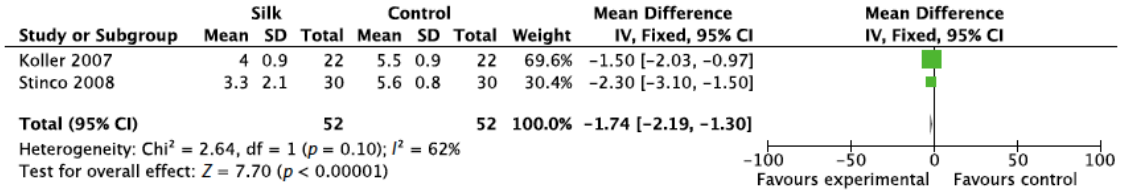

Figure 6 *Meta-analysis of atopic dermatitis symptoms results (silk functional textiles versus placebo).*

5.5.3 Quality of life

Quality of life in patients with AD was assessed using different tools. Gauger et al. (89), using the German Instrument for Assessment of Quality of Life in Skin Diseases (DIELH), showed an overall improvement in quality of life among patients who wore silver-coated garments, but they did not detect any significant differences with patients who wore untreated cotton garments. Kurtz et al. (101), using a study-specific quality of life index, assessed every 2 weeks up to 8 weeks, saw a progressive improvement in quality of life in patients who used silk-like bedding, but there was no comparison with controls.

5.5.4 Rescue medication

The use of rescue medication (topical corticosteroids) was addressed only in studies evaluating silver textiles. Juenger et al. (93), using data from the first 2 weeks of the trial, analyzed the use of prednicarbate ointment (measured in grams) as rescue medication in 3 groups (those who used silver textile, those who used silver-free textile, and those who used prednicarbate ointment regularly), and found that the quantity of rescue medication used by patients in the silver group was similar to that used by the regular steroid group and higher than that used in the silver-free group. In the study by Gauger et al. (89), the percentage of patients who needed topical steroids was 16% lower in the group that wore silver-coated garments than in the group that wore cotton garments.

5.5.5 Skin microbiological profile

The effect of interventions on the skin microbiome was evaluated in terms of *S. aureus* colonization (mean number of colony forming units [CFUs] per cm^2). Of the three studies analyzing silver textiles (90, 93, 95), the two RCTs (93, 95) showed a significant reduction in *S. aureus* colonization. In a case-control study of a silk fabric coated with AEGIS, a nonsignificant reduction in CFUs was seen in both cases and controls (97).

5.5.6 Skin physiology

Skin physiology was assessed by transepidermal water loss (TEWL) in 3 studies: 2 involving silver (94, 95) and 1 involving borage oil (91). In a side-by-side comparison study, compared with placebo, a significant decrease in TEWL was detected after 4 weeks in patients who wore a silver-loaded fiber (95). In the other study of silver, similar results were obtained for mildly involved skin, but not for skin with more severe disease (94). In the borage oil study, TEWL decreased in the study and control groups, but the differences were not significant (91).

5.5.7 Safety

The systemic absorption of silver through the skin in patients who wore fabric impregnated with silver was evaluated by urine and serum silver measurements in 2 studies (93, 95), with no persistent increases detected. In a study of an antimicrobial silk fabric by Senti et al. (99), one of the patients dropped out at day 4 due to a flare in both treated and untreated skin areas.

5.6 Impact of a chitosan coated textile in AD (Study V and VI)

5.6.1 Efficacy and safety of chitosan coated garments

After the 8-week intervention period there was a significant improvement in SCORAD from baseline for both the chitosan group and the placebo group (improvement of 43.8%, 95% CI: 30.9 to 55.9; *p* =0.01 vs. 16.5%, 95% CI: -21.6 to 54.6; *p* =0.02). The respective absolute reductions in SCORAD scores were from 44.2 (95% CI: 34.5 to 53.9) to 29.4 (95% CI: 21.4 to 37.4) and 41.4 (95% CI: 34.3 to 48.6) to 25.7 (95% CI: 18.3 to 33.1); (**Figure 7**). No significant differences were observed between groups for changes in SCORAD.

The improvement in DLQI scores from baseline was 36% (95% CI: 23.5 to 48.1) in the chitosan group (8.0 [9.3-6.7] to 4.8 [6.2–3.4], *p* = 0.02) and 25% (95% CI: 6.0–44.1) in the placebo group (8.3 [10.4-6.3] to 5.6 [7.7-3.5], *p* = 0.28) (**Figure 7**). There were no significant differences between both groups. The proportion of individuals with a clinically meaningful improvement in SCORAD was 25 (67%) in the chitosan group and 20 (63%) in the placebo group. No significant effect was observed either on daily pruritus or sleep loss scores (**Figure 8**), need for rescue medication, or number of flares or totally controlled weeks and well controlled weeks (**Table 8**).

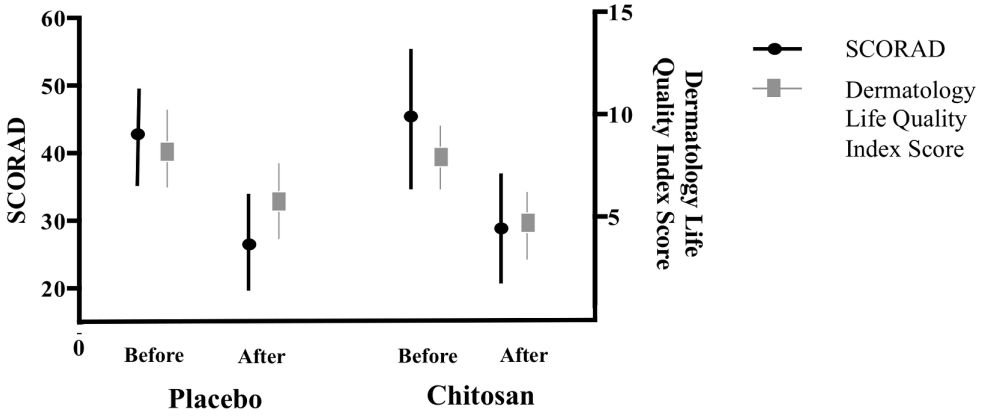

Figure 7 *Mean SCORAD and Dermatology Life Quality Index scores (95% CI) in chitosan and placebo groups before and after intervention*

Table 8 *Differences in efficacy outcomes in chitosan and placebo groups after intervention*

	Chitosan	Placebo	p-value for difference§
Rescue medication, days	2.0 (0.0-8.3)	5.0 (0.0-15.5)	0.82
Flares	0.0 (0.0-1.0)	0.0 (0.0-1.0)	0.73
Totally controlled weeks	4.0 (0.8 – 7.0)	4.5 (1.8-8.0)	0.43
Well controlled weeks	1.5 (0.8-3.0)	2.0 (0.0-3.0)	0.82
Uncontrolled weeks	1.0 (0.0-4.3)	1.0 (0.0-5.0)	0.94

Median (interquartile range) § Mann Whitney test.

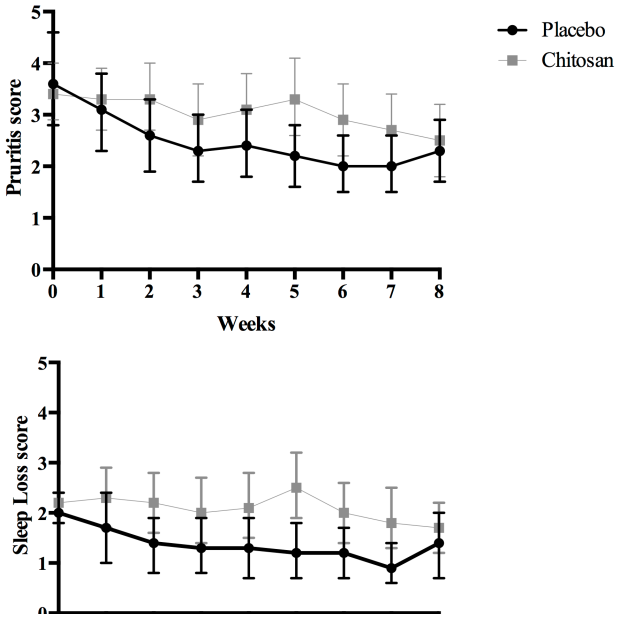

Figure 8 *Mean (95% CI) weekly pruritus and sleep loss scores in chitosan and placebo groups throughout the intervention period. There was a reduction in both groups with no significant differences,p=0.7 mixed effects model*

Most patients had identification of staphylococci species in at least one sampled region with no significant changes after the intervention or for changes between groups (**Table 9**). There was a decrease in the percentage of patients with identification of *S.aureus* from 68% to 55% in chitosan group, in contrast with an increase in the placebo group (from 53% to 64%) that

was not statistically significant. The mean proportion of *S.aureus* counts versus total staphylococcal counts showed no significant differences after intervention for both groups on the five sample regions (right arm, left arm, right leg, left leg, neck) (**Table 9)** neither when considering all regions (**Figure 9**). When considering total bacterial counts there was a significant increase in the mean total staphylococcal count in the chitosan group (*P* = 0.02), with no other differences (**Figure 10**).

Table 9 *Skin microbiological changes after intervention in both groups*

	Chitosan			Placebo			Chitosan vs. Placebo
	Before (N = 38)	After, (N = 34)	P-value	Before, (N = 30)	After (N = 28)	p-value	p-value
Staphylococci +, n (%) of patients	34 (85)	30 (75)	0. 71§	26 (87)	23 (82)	0.92 §	0. 72†
S. aureus +,n (%) of patients	27 (68)	22 (55)	0.92 §	18 (53)	18 (64)	0.72§	0.73†
% CFU S. aureus/total staphylococci							
Right arm	67 (54–81)	60 (45–75)	0.43*	77 (61–91)	83 (71–96)	0.21*	0.14§
Left arm	63 (48–78)	67 (53–81)	0.94*	67 (50–84)	75 (59–70)	0.42*	0.34§
Right leg	68 (54–83)	72 (57–86)	0.32*	70 (55–86)	76 (62–91)	0.52*	0.92§
Left leg	71 (57–85)	70 (57–85)	0.91*	75 (61–90)	73 (59–87)	0.83*	0.73§
Neck	64 (40–80)	48 (32–64)	0.11*	73 (55–89)	87 (28–146)	0.34*	0.93§

CFU-colony forming units.Mean (SD) unless stated otherwise * Wilcoxon Ranked sign test [P] Man Whitney analysis [§]MacNemar test

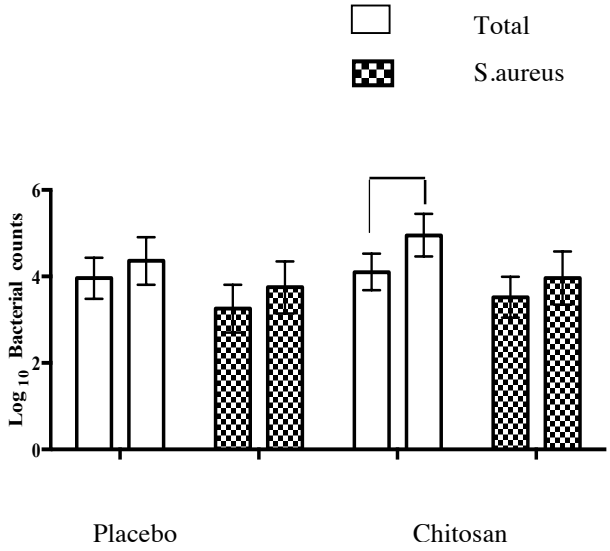

Figure 9 *Mean (95% CI) Log_{10} total staphylococci and Log_{10} Staphylococcus aureus counts for all regions sampled in chitosan and placebo groups before and after intervention. *P = 0.01, Wilcoxon Ranked sign tests.*

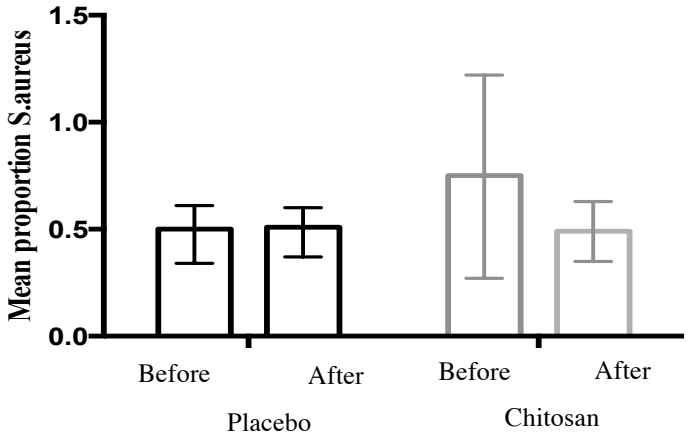

Figure 10 *Mean (IC 95%) proportion of CFU of* S.aureus *vs total staphylocci before and after intervention for placebo and chitosan groups, when considering all regions. There were no significant differences when comparing changes between groups, p=0.29 Man-Whitney test*

The chitosan-coated pyjamas were well tolerated. One patient in the chitosan group decided to withdraw at week 4 due to an AD flare, but no causal link was established.

5.6.2 Immunoallergic modulator effects of chitosan coated garments

With the exception of the increase on eosinophil cationic protein in chitosan pyjamas users (p=0.025), no other differences were seen between groups for specific enterotoxins, total IgE and Phadiatop (**Table 10**).

There was a significant decrease in total IgE and Phadiatop levels in both groups after the intervention but no differences in levels of spIgE to enterotoxins. There was an increase in specific IgE to Malassezia in both groups that reached statistical significance only in placebo group (**Table 10**)

Table 10 *Differences in immunoallergic parameters for atopic dermatitis patients in chitosan intervention (N = 43) and placebo groups (N = 35)*

	Chitosan			Placebo			Chitosan vs. Placebo
	Before	After	p-value¶	Before	After	p-value¶	p-value
Total IgE, UI/ml	5215 (2135 -8284)	3591 (1437 -5746)	0.012	2238 (790 -3686)	1886 (540 to 3232)	0.001	0.721*
Phadiatop, KUA/L	635 (255 - 1014)	475 (209 - 742)	0.001	309 (142 -475)	263 (107 to 419)	0.001	0.643§
Specific IgE, KUA/L							
SEA	5.6 (-1.5 - 12.9)	3.3 (-0.16 - 6.8)	0.811	1.71 (0.57 -2.8)	1.7 (0.4 - 2.9)	0.622	0.522 §
SEB	2.95 (0.6 - 5.4)	3.7 (-0.5 to 7.8)	0.142	1.78 (0.6 -3.01)	1.6 (0.1 - 3.0)	0.943	0.313 §
SEC	3.2 (1.4 - 5.0)	2.9 (1.7 - 4.1)	0.932	3.3 (1.8 - 4.7)	4.6 (0.7 - 8.4)	0.951	0.874§
TSST	2.68 (-0.1 - 5.5)	4.3 (-1.7 - 10.5)	0.121	0.96 (0.4 -1.53)	1.2 (0.5- 2.0)	0.911	0.272§
M.furfur	8.6 (1.6 - 15.6)	12.7 (2.9 - 22.4)	0.172	8.4 (2.7 - 14.1)	14.9 (3.8 - 25.9)	0.042	0.132§
ECP, µg/L	28.6 (23.2 -34.1)	41.5 (28.9 - 54.1)	0.001	33.8 (23.6 - 44.0)	28.6 (23.2 -34.1)	0.632	**0.025***

SEA:enterotoxin A; SEB:enterotoxin B; SEC:enterotoxin C, TSST: Toxic shock syndrome toxin ECP-eosinophil cationic protein SEA-staphylococci enterotoxin A, SEB- staphylococci enterotoxin B, SEC-staphylococci enterotoxin C, TSST-toxic shock syndrome toxin, M.furfur-malassezia furfur ¶ Wilcoxon ranked signs test for dependent samples ; *ANOVA test with t Baseline as covariate, intervention as fixed effects § Mann Whitney U test for independent samples

6. Discussion

6.1 Methodological considerations

The most significant limitation of **Study I** is the small number of patients included. One of its strengths is the comparison with healthy controls, while another is the novel polymerase chain reaction technique used for microbial characterization. The possibility of detailing the molecular genetic profile of bacteria is an important step forward.

The limitations of **Study II** are as follows. First, the absence of healthy control subjects only allows us to speculate on the role of this SNP in patients with a diagnosis of AD. Nevertheless, it was our objective to study the relation of this SNP with bacterial burden in patients, not as a risk factor for AD, where it would have been mandatory to include controls. Secondly, the unknown prevalence of *FLG* mutations in the Portuguese AD and general population. However, results for sample size calculations showed that 42 patients were needed to detect a significant difference in SCORAD score and we were able to include more patients to overcome the level of uncertainty regarding the prevalence of genetic mutations. Importantly, this is the first study relating the presence of p.Pro478Ser to AD severity and bacterial load and its occurrence in European patients with long term AD

The association between psychological traits and AD severity (**Study III**) is limited by its cross sectional nature which does not allow us to establish a causal relationship. A selection bias may had occurred since the recruitment was by invitation and advertisement in media: patients that were available to participate were more prone to extraversion and this may have affected the results of personality traits. The fact that no study has yet been published comparing the 5-main domains of personality assessed by NEO-FFI and their relation with AD severity is one of the strengths of this study. All the used psychological instruments such as the depression, anxiety and personality questionnaires were validated for the Portuguese population. Gender and disease duration that may interfere with psychological analysis were taken in consideration and used as adjusting factors in our analysis. Nevertheless the inclusion of more patients could have elicited different results.

In common with all meta-analyses, **Study IV** may had included studies in which the interventions and characteristics of the human subjects were dissimilar for comparison, resulting in questionable conclusions on the used functional textile. The main problem was the wide range of reported outcome measures regarding AD severity: SCORAD and its adaptations, Eczema Area Severity index and non validated scales of physician and patient rated symptoms We aimed to compose clinically useful and comparable outcome categories from the trials. Measurements of skin microbiological counts were also reported differently. Most studies did not made comparison between intervention groups making it more difficult to interpret reported results.

In **Study V**, the randomisation was successful, as baseline differences among groups were not significant. Allocation concealment was guaranteed until the end of the intervention. The power analysis was based on the minimally clinically important difference of the SCORAD score previous published, and we were able to reach an adequate sample size to detect meaningful differences**.** Only validated outcomes were used and in line with the recently published recommendations of International Consensus Meeting to Harmonise Core Outcome Measures for Atopic Eczema / Dermatitis Clinical Trials (138). Due to the fact that this was an exploratory study and no previous *in vivo* data is published, the exact time of contact between skin and the textile to optimize chitosan antibacterial properties was not known. We considered the use of the functional textile only at night, aiming to target a critical period with more impact on pruritus and sleep loss symptoms (more prolonged skin contact with chitosan may have elicited a more pronounced effect). Patients were also allowed to use rescue medication that could have influenced the treatment effect. However, this was corrected with the mixed model effect used in statistical analysis and the effect on the clinical outcomes analyses should be minimal. I

n AD, skin manifestations are strongly influenced by psychological factors and in this trial the magnitude of the placebo effect could have masked the true differences between treatment groups. The inclusion of patients with different AD phenotypes (IgE mediated and non IgE mediated), colonized and non-colonized with *S.aureus* may have influenced efficacy. Although the analysis of efficacy for these subgroups (data not shown) did not show different results, the targeting of specific populations with more patients could have elicited other results. Importantly, this trial was the first to evaluate the utility of a biopolymer - chitosan - in patients with atopic dermatitis. It is also the RCT evaluating the efficacy and safety of a functional textile in AD with the highest number of included patients.

In this trial we also evaluated the proportion of *S.aureus* versus total staphylococci counts, approaching a more recent concept that more relevant than the isolated counts of *S.aureus* is its dynamic relation with the staphylococcal skin community (31) Our trial included only adolescents and adults patients with long term disease, patients included were stable, leaving little room for improvement. However, our strategy – intervention with chitosan in a concentration known to carry no risks of adverse events and during relatively short term period is more prone to increase compliance in real life.

In **Study VI,** sample size calculations were based on a previous randomized clinical trial with probiotics assessing significant changes in ECP serum levels in AD patients (136). It was considered that probiotics had a similar effect power on intervention to chitosan. This choice may me arguable, but considering this a pilot study, no previous data regarding inflammatory serum markers and chitosan was published.

6.2 Diversity profile from the staphylococcal community on atopic dermatitis skin: molecular approach (Study I)

In this cross sectional study, with a multiplex PCR that allowed the identification and discrimination of

bacteria belonging to the Staphylococcus genus isolated from the skin of patients with AD, we found that Staphylococcus species were present in very high numbers (10–100 times larger than that of healthy controls) and with predominance of two species (*S.aureus* and *epidermidis*). Moreover, we were able to identify that most *S. aureus* strains showed the presence of toxin genes. It was also a novelty to report that *S.hominis* seem to be an important microorganism in AD.

The simultaneous prevalence of *S. aureus* and *S.epidermidis* in our AD patients provided new insights into the relationship between staphylococci, as reported recently (139). These staphylococcal species may share a mutualistic or commensal relationship to enhance common resistance to antimicrobial peptides (140) or enhance binding to exposed extracellular matrix proteins in inflamed AD. Alternatively, the observed concordance may represent a compensatory or antagonistic mechanism of *S. epidermidis*, increasing in an attempt to control *S. aureus*(139). *S. hominis* species is not usually reported as an important microorganism in AD, but in our study they constitute a relevant fraction of the isolates (28%), which possibly could reflect new approaches into the association between staphylococci species similar to those described above for the *S. epidermidis* species.

When comparing the staphylococcal community of patients with healthy controls, differences in the predominant strains and in the diversity of the skin microflora were seen, with nine species being detected in healthy individuals in contrast to four identified in AD: these differences may suggest an adaptation of these strains to the AD environment. Kong et al.(141) also demonstrated in their study of the skin microbiome in children with AD, that increases in *S. aureus* accounted for reductions in skin microbial diversity. However, a cautious note should be made due to the lower number of isolates per individual, increasing the inter-personal heterogeneity, which could in part influence the higher biodiversity of normal skin.

Using the PCR assay, we found that most of *S. aureus* strains showed the presence of toxin genes. Previous studies had shown that more than 50–60% of *S. aureus* strains isolated from patients with AD were exotoxin-producing strains, which can secrete various exotoxins including, e.g., SEA, SEB, SEC, and SED, and TSST-1 (142). We found that more than one toxin gene was detected in all strains up to seven toxin genes detected in one strain. The SEIM and SEIN genes were the most frequently detected, followed by the genes for SEG and SEIO. Those genes are components of the enterotoxin gene cluster (egc) and the previously known egc type (SEG, SEI, SEIM, and SEIN with SEIO or SEIU), (143) was frequently distinguished in our data. These findings are in accordance with other studies that also revealed a high prevalence of SAg genes associated with the egc locus in *S. aureus* isolates from patients with AD (144). Although SEI and SEG are encoded on the same pathogenicity island, the egc cluster, frequently only SEG was detected using the primer pairs as described previously (143) (this effect may be caused by polymorphisms that were found in the egc cluster) (144). Nevertheless, the SEA , SEIL and SEIU genes were also detected but in lower frequencies.

Some authors proposed (142) that the diversity in toxin detection in atopics is associated to the severity of the disease and the site of the skin involved or sampled. This was also confirmed by Yagi and coworkers (145) in a study where *S. aureus* strains were isolated from different skin areas in AD

patients, finding that 41% in the non-lesional area, 62% in the dry-lesional area, and 75% in the exudative-lesional area were SAg positive. Despite the proportion of toxin-producing strains and the frequency of certain toxins differing between studies (146) generally, the SEB or SEC are the most predominant SAg genes detected in AD(142). In our samples none of these two types of SAg genes were detected, which may be attributed to the limited number of isolates and/or individuals we studied. However, Arkwright et al. (147) had also demonstrated that the SEB was poorly detected (4%) in the skin of children with atopic dermatitis.

The main limitation of our study is the limited number of included patients. Its strength is the use of multiplex PCR for characterizing the skin microbiologic profile both in AD patients and controls and not only for determining toxin genes profile as in previous studies (148),(149).

We emphasize that this molecular-based approach successfully identified the staphylococcal microflora that was relatively specific to patients with AD. Therefore, identification and fluctuation of certain micro-organisms and their association with AD reveal the importance of these microbes in the course of human disease. The presence of SAg genes among coagulase-negative Staphylococcus and other SAgs should be further studied to obtain a more comprehensive profile in patients with AD. Further studies with larger number of patients and with a longitudinal study design are needed.

6.3 Relation between FLG genetic profile, skin colonization with *S.aureus*, immunoallergic markers and disease severity (Study II)

In this cross-sectional study we found that the *FLG* p.Pro478Ser polymorphism is significantly associated with more severe disease and higher skin colonization with *S.aureus* in AD patients, in contrast with *FLG* null-mutations. The 478 serine residue may increase skin permeability, leading to higher skin penetration of bacteria and conferring susceptibility to AD (150). Another possible explanation is that an unrecognized functional mutation may be present at or adjacent to the *FLG*, which is in linkage disequilibrium with the P478S polymorphism, thereby contributing to the risk for AD (151). Our findings therefore raise the hypotheses that this SNP may have clinical evident implications of increased skin bacterial colonization and more severe disease in AD patients.

Importantly, this is the first study relating the presence of p.Pro478Ser to AD severity and bacterial load and its occurrence in European patients with long term AD. Only three previous studies have been published regarding this SNP, both in Asian patients, suggesting it confers susceptibility to AD particularly in patients with high IgE levels (152-154). The low prevalence of *FLG*-null mutations in our study is in concordance with its wide variation across the globe and a lower prevalence in Southern European countries; the lack of association with clinical, microbiological and allergic parameters reinforces the fact that other genetic markers in addition to *FLG* mutations should be studied.

The limitations of this study are as follows. First, the absence of healthy controls restricts us to speculation on the role of this SNP in patients with AD. Nevertheless, our objective was to study the association between this SNP and bacterial load in patients and not the role of the SNP as a risk factor for AD, in which case it would have been mandatory to include healthy controls. Second, the

prevalence of FLG mutations in the Portuguese population as a whole and in AD patients in Portugal is unknown. However, the sample size calculations showed that 42 patients were needed to detect a significant difference in the SCORAD score, and we were able to include more patients to overcome the level of uncertainty regarding the prevalence of genetic mutations.

In conclusion, genetic factors can affect the severity of AD and skin microbiota. Our study shows that the presence of p.Pro478Ser may be related to both increased disease severity and bacterial colonization in patients with long-term AD.

6.4 Psychological factors and AD (Study III)

In this cross-sectional study, we found that personality traits may impact on disease severity and that the main determinant of quality of life in long term AD patients is the objective disease burden.

Regarding personality and in contrast with results from a previous experimental setting (60), we found that high conscientiousness was associated with less severe AD. This personality trait is associated with being methodical, hardworking, efficient and organized, focused on solving tasks effectively and results-oriented. Conscientious people tend to be more self-disciplined and more self-controlled(155). Personality traits and coping strategies can influence physical and mental health(156). Coping strategies can be explained as all the strategies adopted by individuals in order to adapt themselves to adverse circumstances, seeking to master, minimize or tolerate stress or conflicts. Previous meta-analyses had linked Extraversion, Conscientiousness and Openness to more engagement coping (aimed at dealing with the stressor in contrast with denial and avoidance). This kind of coping is particularly effective in reducing ling term distress, which may be especially important in in chronic diseases such as AD persisting into adulthood. Given that we had higher Conscientiousness scorers, this personality trait may had enhanced treatment compliance and consequent disease improvement trough more adequate coping strategies as anticipating predictable stressors and avoiding impulsive actions(157). We had also observed that patients scoring low in Extraversion and high in Neuroticism tended to have higher SCORAD mean values. We hypothese that higher scorers on neuroticism are emotionally unstable and low scorers on extraversion are characterized by withdrawn(156) with tendency to inadequate coping strategies.

In terms of psychological distress, we found slightly lower levels of anxiety in our sample (13.6%) when compared with other international surveys (17.6%) and higher than healthy controls (11.1%)(61) but anxiety levels didn't seem to affect disease severity. This is also consistent with previous studies evaluating AD severity with a composite score as SCORAD (60, 64, 158).

Regarding depression, we found lower levels (2.3%) than in other AD (10.1%) and healthy controls (4.1%) samples (61). We cannot exclude that a larger sample would have elicited different results. Nevertheless, for the few patients reporting depressive symptoms there was a significant relation with increased AD severity in line with previous studies(60, 64, 158). More severe disease with more physical discomfort can imply hopelessness that is one of the most important depression's symptoms.

Moreover, depressed patients may also neglect their treatment leading to disease worsening..

We also found a significant relation between SCORAD and quality of life. Quality of life comprises factors such as physical, functional, emotional and intellectual well-being, work, family and friends. Most authors agree that factors affecting QOL should be divided into two groups: subjective and objective(62). The subjective factors include self-assessment of physical condition, mental condition (anxiety, depression, self-esteem); social situation (e.g. satisfaction with job) and interpersonal relations (e.g. social support). The objective component of QOL refers to medical diagnosis. In our study, the assessment of QOL was done through DLQI that specifically assess functional lifestyle quality of life in relation to work, leisure, relationships, daily activities and treatment. Although it is not an AD specific questionnaire, it has been widely used in AD related research(63, 65, 159, 160).

The absence of a significant relationship between personality/psychological symptoms and DLQI, and the fact that disease severity was the main determinant of QoL points out that the most important factor affecting patients well being was the objective disease burden. This is not consistent with previous studies that showed a controversial relation between anxiety and QOL and that depression seemed to predict lower QOL (63, 64). In those studies a more heterogeneous sample in terms of degree of AD severity was evaluated which may have contributed to this discrepancy. These results highlight the importance of achieving complete control of skin symptoms, disease intensity and extension to improve patients well being.

Regarding study limitations, we can refer primarily the inability to assess the causal directionality of the associations given its cross sectional design. Secondly, the limited sample size, although our study had a similar number of included patients to the two previous studies addressing this subject (60, 161). A bias in the participant selection may have also occurred, resulting in enrolling patients with low depression scores and high sociability.

Our study has some important strengths: it was carried on an outpatient setting with validated clinical outcomes as previously suggested by other authors(60); we had applied the same psychological questionnaires that had been used on a recent European Multicentre survey(61) and that were previously validated in the Portuguese population(126) facilitating future comparisons.

Therefore, psychological assessment and intervention with characterization of personality profile and adequate education and training of adaptive coping strategies may benefit patients with long term atopic dermatitis. In patients with more severe disease the importance of depressive symptoms should be highlighted and achieving total disease control is decisive.

6.5 Recommendation for use of functional textiles in AD (Study IV)

Our systematic review found that the use of functional textiles in atopic dermatitis is safe and associated with a slight improvement in disease severity, symptoms, and quality of life. However, any recommendations for the use of these textiles as part of standard AD management are hampered by the low quality of supporting evidence. Different textile components are associated with different effects. Silver-coated cotton, for example, seems to be more effective in decreasing lesion severity,

while silk fabrics appear to be more likely to alleviate pruritus and symptoms.

The evidence for the effectiveness of functional textiles in AD was qualified using the GRADE approach. In addition to an overall lack of evidence supporting the use of functional textiles in AD, the quality of evidence in the studies included in our review was either low or very low, mainly because they were non-randomized, non-controlled studies, which furthermore were underpowered to detect treatment effects due to small sample sizes. Short follow-up might also have reduced the ability to see true effects, possibly explaining why some studies did not detect differences between placebo and intervention groups. The use of different textiles, with different active compounds and therefore different physical and antimicrobial properties, prevented direct comparisons between studies. Accordingly, we only performed a meta-analysis of studies that evaluated the same interventions and outcomes. The limitations of this review are explained by the limitations of the studies included.

All the studies analysed in this review that addressed eczema severity reported some benefits from using functional textiles, but the majority did not compare results with those from a control group. Due to differences in study design, interventions, and outcome measures, we were only able to pool data on SCORAD in 2 studies (89, 93), both of which analysed silver-coated textiles. The meta-analysis showed a trend in favour of the use of these textiles.

Silver seems to exert its effect on eczema severity through its antimicrobial properties (162), diminishing colonization by *S. aureus* and consequently attenuating inflammation and consequent exacerbation of lesions. Nevertheless, definitive conclusions cannot be drawn, as we analysed only 2 studies, with different designs and small sample sizes.

Silk textiles may affect overall disease status by improving comfort and reducing itch sensation. Almost all the studies of silk analysed in this review used specific types of silk fabrics made of transpiring and slightly elastic woven silk, free of sericin (a protein assumed to be irritant to the skin), and impregnated with AEGIS, an antibacterial compound (96-100). The exception was the study by Kurtz et al. (101) which did not state which antimicrobial was used. Silk did not have a significant effect on *S. aureus* colonization, although this was analysed in just 1 study (99). The use of silver textile, however, was significantly associated with a reduction in *S. aureus* colonization; the difference in effects may possibly be due to different mechanisms of action.(162) The use of EVOH fiber in AD is intended to reduce pruritus, as fabrics treated with EVOH have a smooth texture. However, in our review, the single study that analysed EVOH fiber reported an improvement only in erythema. Borage oil has been previously used in AD to restore skin barrier lipids as an oral supplement, with conflicting results (163, 164). The lack of comparison with placebo in the study analysing borage oil–coated textiles in our review (91) made it impossible to draw any definitive conclusions on effectiveness.

Functional textiles used in AD are designed not only to reduce disease severity, but also to alleviate symptoms. In most cases, the aim is to improve pruritus and sleep loss, two of the most distressing features of AD. Most of the studies we reviewed reported improvements in pruritus and sleep disturbance following the use of specially treated fabrics, but, in half of the studies, no between-group

comparisons were made. The use of silver-impregnated cotton fabric with an antimicrobial effect may contribute to the relief of symptoms. The 2 studies that analysed silk reported a significant decrease in symptoms, and the meta-analysis of pooled data suggested that this fabric might be effective in improving the symptoms of AD. However, due to the small number of studies and small sample sizes, a definitive conclusion cannot be drawn.

A reduction in symptoms and colonization by *S. aureus* may also have an impact on quality of life. Nevertheless, the different tools used to measure this outcome—and the different study designs—prevent any conclusions from being made. The need for rescue medication was addressed in 2 studies (89, 93) but the results are not comparable as different outcomes were used (quantity of medication used and percentage of participants requiring medication)

The impact of the use of functional textiles on the skin microbiome was evaluated in only 4 studies (90, 93, 95, 97), even though a reduction in skin colonization by *S. aureus* is one of the aims in the use of functional textiles. Beneficial results were seen only with silver, which is understandable given its antimicrobial properties, but no conclusions can be drawn due to the low quality of the supporting evidence. Measures of skin physiology are also important when evaluating skin inflammation. Improvements in TEWL may result from a reduction in skin inflammation associated with a reduction in pruritus and bacterial colonization favoured by the use of functional textiles. In our review, we detected conflicting results in the study by Park et al. (94), which showed less or no TEWL improvement in patients with more severe forms of AD.

Although the studies included in this review analysed different populations, age groups, and degrees of disease severity, only one adverse event—an eczema flare-up—was reported. The event, however, could not be directly linked to the intervention (use of antimicrobial silk fibre), since both treated and untreated areas were affected (99).

The methodological quality of future studies of functional textiles in AD needs to be improved in order to enable similar outcomes to be analysed across different textiles. The emergence of new compounds may also offer improved effectiveness (165). An appropriate sample size should be calculated according to the evaluated outcomes and type of study design .The possibility of targeting specific AD phenotypes (166) (e.g. *S. aureus* colonization, atopic versus non-atopic, presence or absence of filaggrin gene mutations) may also improve the performance of certain textiles in subgroups of patients. The role of functional textiles in AD needs to be addressed by more studies, with longer follow-up and an improved design.

6.6 Effect of chitosan coated textiles in AD (Study V, VI)

6.6.1 Efficacy and safety

In this randomized controlled trial, chitosan coated textiles, used for 8 weeks, were associated with a non-significant trend of disease severity improvement. Moreover, this effect was related with a significant increase of skin coagulase negative Staphylococci.

Our study has some limitations. First, since this is a pilot study, the number of participants and outcomes assessed may have been not sufficient to detect significant differences. However, based on previously published minimally clinically important differences for SCORAD, the study was designed to be sufficiently powered to detect meaningful differences. However, post hoc analysis evaluating the high range of confidence limits in the control group versus the active one suggested this may have not been the case. Furthermore, we only used validated outcomes in line with recently published recommendations (138).

Second, the study participants were adolescents and adults with long-standing atopic dermatitis and there would probably be a greater likelihood of detecting clinically significant improvement in adults with more severe disease.

Thirdly, because no *a priori* data exist on the duration of the intervention and its *in vivo* effects, we cannot rule out that longer skin contact with chitosan might have elicited a more pronounced effect. However, the participants were instructed to wear the pyjamas every night for the duration of the study, as we wished to target a critical period. Finally, the fact that the patients were allowed to use rescue medication may have influenced the effect of the intervention. However, this was corrected for in the mixed effects model and the effect on clinical outcome analyses should therefore be minimal. This is the first trial to evaluate the utility of a biopolymer in patients with AD and, so far, it is the largest study of functional textiles. Another innovative aspect was the evaluation of other staphylococcal species than *S.aureus* (31).

Chitosan has exhibited skin repair potential in wounds and antimicrobial action in diverse medical fields (167-170), explaining why chitosan could potentially improve disease severity in patients prone to non-commensal bacteria colonization and skin barrier impairment. In the present study, chitosan-coated garments had no effect on the skin *S.aureus* counts but surprisingly, we observed in the chitosan group an increase in total staphylococci counts independently of *S. aureus,* corresponding to coagulase negative staphylococci species (CNS). The increase of CNS on the skin of AD patients has been already reported eliciting different explanations for this fact: some authors argue that it may be the result of a mutualistic relationship or represent a compensatory or antagonistic mechanism to control *S.aureus* (171). Our data supports the hypothesis that chitosan may had exerted a specific inhibitory effect upon *S. aureus*, allowing the proliferation of other staphylococcal species. Nevertheless, the clinical significance of this observation is exploratory.

The observed placebo effect on disease severity may possibly be due to the improved skin comfort provided by the long-sleeved organic cotton pyjamas used, and/or to the patients' enthusiasm about participating in a clinical trial with a new product.

The significant improvement on quality of life with chitosan treatment was probably related to reduction in AD severity in this group. Considering that sample size was calculated to detect changes in SCORAD index, we can not exclude that more patients were needed to elicit a more pronounced effect on this outcome.

The intervention was well tolerated over the 8-week study period. There was one moderate adverse event, deemed to be unrelated to treatment, in the chitosan group. Safety of functional textiles is a controversial issue since some authors have claimed that the use of antimicrobial compounds could remove bacteria from the skin surface and pave the way for invasion by pathogenic bacteria, such as methicillin-resistant *S. aureus* (172).

6.6.2 Immunoallergic effects

In this randomized controlled trial, chitosan coated textiles, used for 8 weeks, we found a significant increase in ECP serum values in chitosan group that was not observed on IgE mediated allergic serum markers.

It has been previously described that chitosan accelerates migration of polymorphonucleares to wound areas, secreting inflammatory mediators such as tumor necrosis factor (TNF - α) and interleukin -1.(173) TNF - α seems to protect eosinophils from apoptosis under inflammatory conditions in *in vivo* mouse models.(174) Since ECP is an indirect marker of eosinophil degranulation, we may hypothesize that chitosan textiles promoted eosinophil survival and activation contributing to increase in serum ECP. The clinical implication of this result is unknown.

Our study has a few limitations. First, because no *a priori*, data exists on the duration of the intervention we cannot rule out that a more prolonged skin contact with chitosan or higher number of included subjects might have elicited a significant effect on IgE markers. Secondly, since patients were included regardless of their atopy status, we can not exclude that specific AD phenotypes could have elicited different results.

Importantly, this is the first study addressing the impact of chitosan textiles in serum immunoallergic markers of AD patients. Our findings suggest that overnight use for 8 weeks of chitosan textiles is associated with increased serum ECP but not IgE mediated allergic inflammation. Further studies are needed to evaluate the clinical relevance of our data.

6.7. Implications for practice and future research

With our study, we were able to demonstrate that the molecular characterization of skin microbiome trough PCR multiplex constitutes an added value to the understanding of AD pathogenesis, that the characterization of FLG polymorphisms in our Sothern European population may be more clinically meaningful than FLG mutations and that psychological characterization aiming at specific psychotherapeutic interventions are important in AD management. Considering the traditional functional textiles, the evidence of recommendation of its use is weak. We also showed that the modulation of the skin staphylococci community trough the use of a functional textile may improve AD severity and potentially quality of life and furthers studies with chitosan coated textiles are needed.before a recommendation for its use can be made

Several issues still require further investigation.

How exactly can the dysbiosis found on the skin of AD patients influence skin inflammation? Can skin microbiota modulate epigenetic alterations in the host? What exactly is the relation between coagulase negative staphylococci and *S.aureus*?

Why only a subset of FLG mutation carriers has AD? Why some AD patients outgrew their disease and other do not with the same FLG mutation? Are these facts linked to the specific skin microbiome of AD patients?

Is there a specific psychological profile of patients with long term AD? How determinant is each patient´s “disease narrative” for AD severity?

Would chitosan elicit different results in different group of patients as children? What are the effects of chitosan in other skin microbiome constituents than the staphylococci community?

Is the increased serum ECP after chitosan coated garments a marker of increased allergic inflammation or antibacterial activity?

7. Conclusions

In the present study, the influence of skin microbiological profile, genetic background, immunoallergic profile and psychological traits on atopic dermatitis were investigated. The clinical and immunomodulator impact of a textile coated with a new biopolymer was addressed. Based on the results presented in this thesis the following conclusions can be drawn:

(1) In AD patients, the skin staphylococcal community is less diverse than in healthy subjects with predominance of *S. epidermidis* and *S. aureus*. Most *S.aureus* strains presented toxin genes and *S. hominis* species have been reported as an important fraction of staphylococci isolates.

(2) Filaggrin loss of function mutations R501X and 2282del4 were not associated with AD severity, *S.aureus* colonization or inflammatory systemic and allergic markers. The polymorphism P478S was associated with more severe disease and increased bacterial colonization in the first study genotyping the FLG gene in a sample of Portuguese AD patients.

(3) The personality trait of conscientiousness meaning self-discipline and aiming for achievement, seems to exert a protective effect on AD. Depressive but not anxiety symptoms were associated with more severe disease.

(4) Different textile components are associated with distinct effects; silver-coated fabrics, for example, seem to be more effective at diminishing the severity of lesions, while silk fabrics seem to perform better in terms of alleviating pruritus and other symptoms. Based on the low quality of evidence supporting the effectiveness of functional textiles in alleviating symptoms and reducing disease severity in AD, the strength of the recommendation to use these textiles in this setting is weak.

(5) Chitosan coated garments may impact on disease severity by modulating skin staphylococcal profile. Moreover, a potential effect in quality of life may be considered

(6) Chitosan textiles used for a 8-week period during the night are associated with increased serum ECP but not IgE mediated allergic inflammation. Further studies are needed to evaluate the clinical relevance of these data.

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Original Publications

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ORIGINAL ARTICLE

A diversity profile from the staphylococcal community on atopic dermatitis skin: a molecular approach

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Abstract

Aims: The aim of this study was to determine the biodiversity of the skin staphylococcal community from patients with atopic dermatitis (AD) and superantigen (SAg) detection from *Staphylococcus aureus* isolates.
Methods and Results: In this study, we developed a novel multiplex PCR that allows the identification and discrimination of bacteria belonging to the *Staphylococcus* genus both *Staph. aureus* and coagulase-negative *Staphylococcus* – *Staph. capitis*, *Staph. epidermidis*, *Staph. haemolyticus* and *Staph. hominis* isolated from the skin of patients with AD. In addition, a multiplex PCR assay that allows the rapid screening of the 19 genes that encode *staphylococcal* enterotoxins (SEs), SE-like toxins and toxic shock syndrome toxin-1 was also performed and applied in *Staph. aureus* isolates. The microflora of the skin of patients with AD was dominated by *Staph. aureus* (69 isolates, 35·6%) followed by *Staph. epidermidis* (59 isolates, 30·4%) species. The SElM and SElN genes were the most frequently detected in our study (15 isolates, 71·4%), followed by SEG and SElO (14 isolates, 66·7%).
Conclusions: Our molecular-based approach successfully identified the staphylococcal microflora that was relatively specific to patients with AD. Considering skin colonization and expression of virulence factors, the *Staph. aureus* may play a relevant role in AD pathophysiology.
Significance and Impact of the Study: This ability to classify disease-related microbial species provides new insights into the relevance of those microbes in human disorders.

Introduction

Skin is a complex and dynamic ecosystem inhabited by a large multitude of micro-organisms. The composition of the human skin microbiota is influenced by host demographics and genetics, human behaviour, local and regional environmental characteristics and transmission events. This variance could definitively influence human health and disease outcomes among individuals. Therefore, the biodiversity profile of the human microbiota may be predictive or diagnostic of some disease states (Rosenthal *et al.* 2011).

The human-skin-resident microflora is mainly composed of the *Staphylococcus*, *Corynebacterium*, *Propionibacterium*, *Micrococcus*, *Brevibacterium*, *Acinetobacter* Genera (*Cogen et al. 2008*).

Atopic dermatitis (AD) is an inflammatory skin disorder, chronically relapsing and genetically linked. The occurrence of AD is 10–20% and 1–3% in children and adults worldwide, respectively (Leung and Bieber 2003). It is the most frequent chronic skin disorder in children, with an increasing incidence and a considerable, unfavourable impact on the quality of life. This is a multifactorial skin disorder, which consists of complex connections among susceptibility genes, ecological factors and allergens, skin barrier dysfunction, and uncharacteristic systemic and local immune reactions (Lin *et al.* 2007; Lopes *et al.* 2011). Mutations in the gene that produces the skin barrier protein filaggrin are associated with AD, predominantly in patients, which consequently develop asthma and/or allergic rhinitis, suggesting that epicutaneous

sensitization may provide atopic disease (Palmer *et al.* 2006; Sandilands *et al.* 2007). These patients may experience repeated skin infections with increased numbers of pathogenic bacteria, such as *Staphylococcus aureus*; however, nonpathogenic bacteria usually present on the skin surface could take part in a protective role. The diminished expression of antimicrobial peptides on patients with AD skin might contribute to this vulnerability (Ong *et al.* 2002). The potential pathogenic *Staph. aureus* is not recognized as a member of the resident skin microflora. In healthy individuals, the frequency of *Staph. aureus* skin colonization is about 5%, whereas the frequency of *Staph. aureus* skin colonization is more than 90% in patients with AD in both the lesional and nonlesional skin: quantitatively, *Staph. aureus* counts could differ by 100- to 1000-fold between lesional skin and nonlesional skin (Lin *et al.* 2007). Moreover, the colonization rate and density by *Staph. aureus* species on lesional skin are also considerably correlated with the clinical severity of AD disease (Leyden *et al.* 1974; Hauser *et al.* 1985; Lin *et al.* 2007). In addition, more than 70% of *Staph. aureus* strains from AD skin are exotoxin producers and are able to secrete various exotoxins with superantigen (SAg) activity (Hoeger *et al.* 1992; Akiyama *et al.* 1996; Lin *et al.* 2007). These exotoxins might go through the skin barrier and contribute to the perseverance and exacerbation of the allergic state (Bunikowski *et al.* 2000). Various studies also indicate a positive correlation between the clinical severity and the colonization by SAg-producing strains of *Staph. aureus* in AD (Lin *et al.* 2007; Zuel-Fakkar and El-Shokry 2010; Nada *et al.* 2012). Thus, a more thorough understanding of skin staphylococcal community in patients with AD may provide a foundation regarding the clinical management of staphylococcal infections in AD. Additionally, to our knowledge, no study has been performed on the skin of the staphylococcal community of Portuguese patients with AD.

To study the biodiversity of the skin staphylococcal community in AD, we used a molecular-based approach, which enabled rapid analysis of the staphylococcal composition from the skin of Portuguese patients with AD. Simultaneously, a multiplex PCR system detecting 19 types of SAg genes, such as staphylococcal enterotoxins (SEs), SE-like toxins (SEls) and toxic shock syndrome toxin-1 (TSST-1), was applied to the *Staph. aureus* isolates.

Materials and methods

Study design

The study population comprised nine patients attending an allergy clinic with medical diagnosis of AD according to Hanifin and Rajka (1980) criteria (aged 3–35 years), with clinically apparent AD lesions without signs of secondary infection, and consecutively included. Patients were not taking systemic antibiotics, topical corticosteroids, calcineurin inhibitors or immunosuppressants during the previous 2 weeks of medical observation and sampling. All patients had moderate-to-severe disease, and their atopy status was characterized. Their baseline characteristics are described in Table 1.

Table 1 Baseline characteristics of participants

Baseline characteristics	Patients with AD	Healthy controls
Total subjects analysed	9	24
Total samples analysed*	18	24
Age, mean (range) (years)	15 (3–35)	22 (19–33)
Male/female	4/9	11/24
Rhinitis	9/9	0
Asthma	6/9	0
Food allergy†,‡	2/9	0
Dust mite allergy†	8/9	0
Pollen allergy†	6/9	0

*Within patients with AD, the samples were obtained from the antecubital and popliteal crease, and in healthy individuals, the samples were obtained only from antecubital crease.
†Confirmed by skin prick tests and/or specific IgE measurements.
‡Peanuts and nuts in one patient and milk and egg allergy in one patient.

Samples from the antecubital crease of 24 healthy controls obtained through a previously reported research (Tavaria *et al.* 2012) were also integrated in this study.

Sample collection

The sampling procedure was performed in 25 cm^2 of the skin area on the sampling sites (popliteal and/or antecubital crease). The skin within the enclosed area was scrubbed using a sterile swab moistened with dilution liquid. The tip of the swab was then broken against the wall of a glass tube containing the dilution liquid, and the tube was immediately capped and shaken to suspend the bacteria. The samples were cultured in Baird Parker medium (BPM; Lab M, Lancashire, UK) by spread plate technique in duplicate and incubated at 37°C during 24–48 h. The micro-organism isolation procedure was performed as described by Soares *et al.* (2011). A total of 194 isolates were obtained from the skin of patients with AD and 48 isolates from the skin of healthy individuals, isolated and selected according to Tavaria *et al.* (2012).

DNA preparation

DNA was isolated as described by Soares *et al.* (2011).

Reference strains

The type strains used in this study were as follows: *Staph. aureus* ATCC 25923, *Staph. epidermidis* ATCC 14990, *Staph. capitis* subsp. *capitis* ATCC 27840, *Staph. haemolyticus* ATCC 29970 and *Staph. hominis* subsp. *hominis* ATCC 27844. In addition, the type strains of *Staph. aureus* that are described in Soares *et al.* (2011) were used as positive controls for SAg genes.

Molecular identification of the staphylococcal isolates

Multiplex PCR
The wild isolates obtained were submitted to multiplex PCR with the primers FemF/FemR (Mehrotra *et al.* 2000), SepF/SepR (Martineau *et al.* 1996), ScapF/ScapR (Iwase *et al.* 2007), ShaemF/ShaemR (Schuenck *et al.* 2008) and ShomF/ShomR (Vannuffel *et al.* 1999) for the identification of the *Staph. aureus*, *Staph. epidermidis*, *Staph. capitis*, *Staph. haemolyticus* and *Staph. hominis* species, respectively. The list of the primers used and the PCR products generated is described in Table 2. Multiplex PCR and amplification were performed as described by Tavaria *et al.* (2012).

sodA amplification and sequencing
Complementary identification of three (or one in the case of *Staph. lentus*) isolates for each species formerly identified by multiplex PCR was performed by sodA gene sequencing as described by Tavaria *et al.* (2012).

Multiplex PCR for the detection of SAg genes
Twenty one of the 69 isolates previously identified as *Staph. aureus* strains from Portuguese patients with AD were screened for SAg gene detection. They were randomly selected and consist of five to six isolates per individual positive for *Staph. aureus*. The primers were first applied individually, and multiplex PCR conditions were prepared as described by Hwang *et al.* (2007).

Other virulence factors
For virulence factor detection, namely coagulase production, DNase and haemolytic activity, strains were tested as demonstrated by Soares *et al.* (2011).

Results

Staphylococcus identification

From the AD subjects analysed ($n = 9$), we obtained six individuals with skin samples positive for *Staphylococcus* spp. as determined by the BPM counts. After preliminary screening for Gram-positive and catalase-positive and to distinguish among staphylococcal species, we generated a multiplex PCR to uniquely classify the isolates.

To ensure a good amplification with the five primer pairs in the same PCR run, the reaction conditions for the multiplex PCR assays were optimized, which resulted in a strong and reproducible amplification with primers used. The specificity of the multiplex PCR was assessed with a panel of five reference strains, and the results of the multiplex PCR amplification are reported in Fig. 1. The five reference strains of the *Staphylococcus* genus were identified as *Staph. aureus*, *Staph. epidermidis*, *Staph. capitis*, *Staph. haemolyticus* and *Staph. hominis* by yielding specific DNA fragments of sizes 723, 124, 208, 271 and 866 bp, respectively. From these 194 isolates, 69 (35·6%) were identified as *Staph. aureus* by yielding a PCR product of 723 bp. The proportion of *Staph. epidermidis* was also significantly higher with 59 (30·4%) isolates identified; 54 (27·8%) isolates were identified as *Staph. hominis*, which gave an amplification product of 866 bp; 12 (6·2%) isolates were recognized as *Staph. capitis* by yielding a PCR product of 208 bp. Thus, four different species

Table 2 DNA oligonucleotide sequences used as primers and expected size of PCR products used in the multiplex PCR for *Staphylococcus* identification

Primers	Oligonucleotide sequence 5′-3′*	Target species	Amplicon bp	Reference
FemF	ACAGCTAAAGAGTTTGGTGCCTxxxxxGATAGCATGC	*Staphylococcus aureus*	723	Mehrotra *et al.* (2000)
FemR	TTCATCAAAGTTGATATACGCTAAAGGTxxxxxCACACGGTC			
SepF	ATCAAAAAGTTGGCGAACCTTTTCA	*Staphylococcus epidermidis*	124	Martineau *et al.* (1996)
SepR	CAAAAGAGCGTGGAGAAAAGTATCA			
ScapF	GCTAATTTAGATAGCGTACCTTCA	*Staphylococcus capitis*	208	Iwase *et al.* (2007)
ScapR	CAGATCCAAAGCGTGCA			
ShaemF	GGTCGCTTAGTCGGAACAAT	*Staphylococcus haemolyticus*	271	Schuenck *et al.* (2008)
ShaemR	CACGAGCAATCTCATCACCT			
ShomF	TGCCATATAGTCATTTACG	*Staphylococcus hominis*	866	Vannuffel *et al.* (1999)
ShomR	GTTCTAATTGAAGTTGTGTTG			

*Underlined base pairs are polydeoxyinosine bridge region.

were recognized because the *Staph. haemolyticus* species was not identified in this study. The cultivation approach has confirmed a higher possibility of *Staph. aureus* colonization in AD skin given that the proportion of *Staph. aureus* detection (4 of 6 patients with AD) was significant as described in Fig. 2.

To assess how distinct the staphylococcal skin flora for AD might be, we compared it with isolates from 24 healthy individuals (Tavaria *et al.* 2012). The samples from healthy individuals were characterized by a greater heterogeneity in terms of the number of identified species, viz. *Staph. aureus* (eight isolates), *Staph. capitis* (four isolates), *Staph. epidermidis* (four isolates), *Staph. haemolyticus* (five isolates) and *Staph. hominis* (four isolates), *Staph. lentus* (one isolate), *Staph. lugdunensis* (three isolates), *Staph. saprophyticus* (nine isolates) and *Staph. warneri* (10 isolates) as seen in Fig. 2.

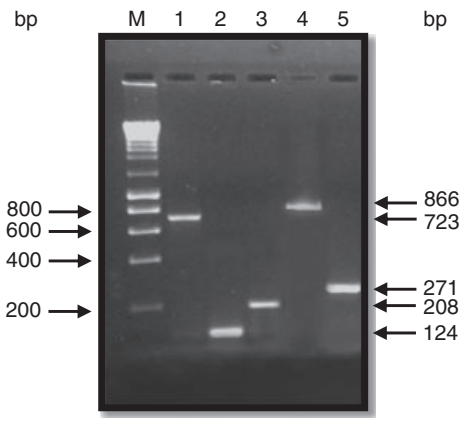

Figure 1 Multiplex PCR amplifications obtained with *Staphylococcus aureus* -, *Staph. epidermidis*-, *Staph. capitis*-, *Staph. haemolyticus*- and *Staph. hominis*-specific primers. Lane 1, *Staph. aureus* ATCC 25923; lane 2, *Staph. epidermidis* ATCC 14990; lane 3, *Staph. capitis* subsp. *capitis* ATCC 27840ATCC 15305; lane 4, *Staph. haemolyticus* ATCC 29970; lane 5, *Staph. hominis* subsp. *hominis* ATCC 27844; and lane M, 200-bp marker NZYTech.

SAg genes detection

The primer sets successfully amplified the target genes in the multiplex PCR without nonspecific or additional bands on the reference strains (data not shown). All of the PCR products showed the expected size when tested individually and were coordinated with the results described by Løvseth *et al.* (2004) and Hwang *et al.* (2007). In this study, 21 *Staph. aureus* strains isolated from AD skin were tested, and sixteen (76%) of them were SAg-positive strains (Fig. 3). The most frequently detected genes were SEG, SElM, SElN and SElO (15 isolates, 71%), and they were always found together in the same isolate, with the exception for one isolate where we did not detect the SElO gene.

SEA (six isolates, 29%) and SElL (seven isolates, 33%) were also frequently detected along with the SEG, SElM, SElN and SElO genes. The classical SEs (SEA-SEE) were not detected in our samples. The SElU gene (one isolate, 5%), which was rarely detected, was also found together with the SEG, SElM, SElN and SElO genes. Based on the SAg genotype of *Staph. aureus* isolates, we obtained six different genotypes with SEG, SElM, SElN with SElO combination being the most predominant (eight isolates) followed by SEA, SEG, SElM, SElN, SElL with SElO combination with three isolates.

Other virulence factors

Sixty-nine (35·6%) and eight (16·7%) isolates among AD and control individuals, respectively, were positive for

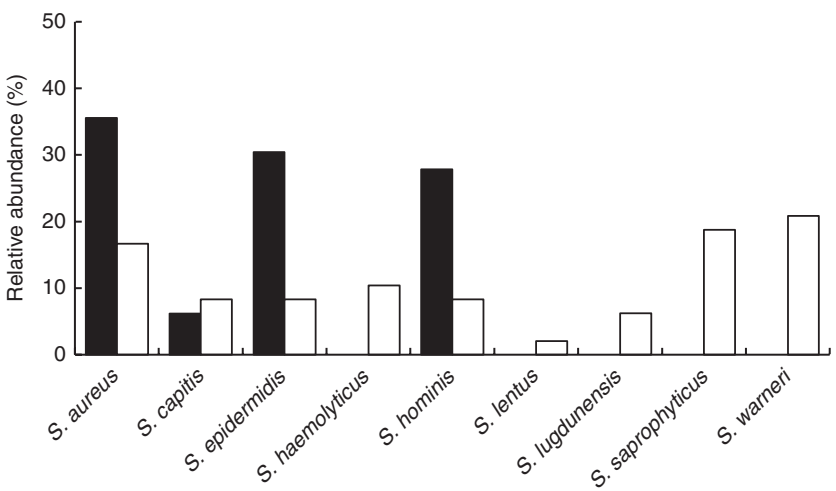

Figure 2 Bacterial taxonomic classifications with mean relative abundance of the staphylococcal species identified from the skin of patients with AD. Non-AD controls are also included, consisting of two isolates per individual from 24 healthy individuals as previously demonstrated by Tavaria *et al.* (2012). (■) AD patients and (□) non-AD controls.

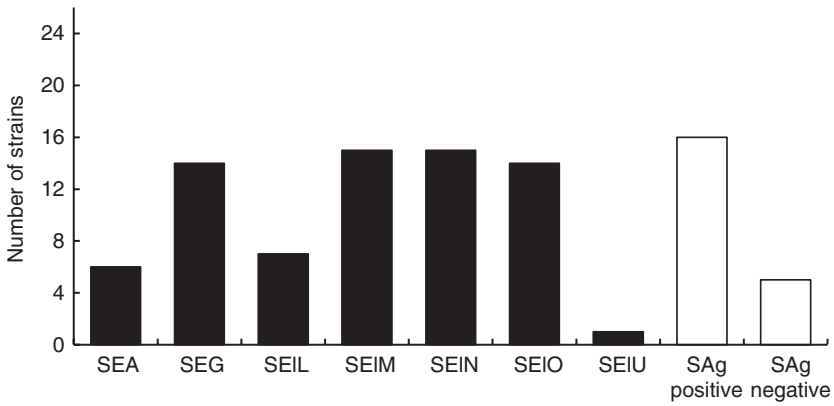

Figure 3 Diversity of SAg genes detected and prevalence of toxigenic and nontoxigenic strains of *Staphylococcus aureus* isolated from the skin of Portuguese patients with AD.

both the presence of coagulase and DNase and correlated with the *Staph. aureus* isolates as expected. In terms of haemolytic activity as performed in horse blood agar, a similar occurrence was seen in control (39·6%) and AD individuals (33·7%).

Discussion

Atopic dermatitis is a frequent inflammatory skin disorder generally associated with the antecubital and popliteal regions, sites that possess identical groups of micro-organisms, but different proportions of such microbial communities (Grice *et al.* 2009). These findings suggest the preferential role of some micro-organisms in skin disorders and their predisposition for conventional sites in human body (Kong *et al.* 2012). AD is typically treated with antimicrobial approaches, and therefore, this is an example of a disorder in which the involvement of micro-organisms in the course of the disease can be assessed by microbial community analyses (Gloor *et al.* 1982; Kong *et al.* 2012).

To classify the staphylococcal population isolated from the skin of patients with AD, multiplex PCR targeting predominant species could be the method of choice for rapid and reliable identification. To our knowledge, no previous study exploited this molecular approach for the identification of staphylococcal strains isolated from the skin.

Using direct multiplex PCR, we defined and characterized the staphylococcal skin community in six patients with AD. To distinguish the five species, which could have a relevant interest for AD, the multiplex PCR-based approach associated five pairs of primers amplifying species-specific fragments designed for *Staph. aureus* (Mehrotra *et al.* 2000), *Staph. epidermidis* (Martineau *et al.* 1996), *Staph. capitis* (Iwase *et al.* 2007), *Staph. haemolyticus* (Schuenck *et al.* 2008) and *Staph. hominis* (Vannuffel *et al.* 1999). These primers were selected based on their thermodynamical compatibility and on the production of PCR bands distinct in size.

From the six individuals positive for *Staphylococcus* spp., as determined by the BPM counts, the staphylococcal microflora was dominated by *Staph. aureus* (69 isolates, 35·6%) followed by *Staph. epidermidis* (59 isolates, 30·4%) and *Staph. hominis* (54 isolates, 27·8%) species in patients with AD (Fig. 2). Although the diminutive study cohort, four patients (67%) were found to be colonized with *Staph. aureus* in their skin. These results were in agreement with those reported by Lin *et al.* (2007), which demonstrated that 75–100% of patients with AD have *Staph. aureus* on their lesional skin. Moreover, a major feature of AD is the presence of *Staphylococcus* species in very high numbers (10–100 times larger than that of normal individuals) (Gloor *et al.* 1982).

The *Staphylococcus* species detected in this study includes both coagulase-negative staphylococci, a major member of normal microbiota, and *Staph. aureus*, a potential pathogen. The genus *Staphylococcus* has several bacterial species relevant to the clinical disease, which includes *Staph. epidermidis* as a skin commensal and *Staph. aureus* as a micro-organism associated with AD (Kong *et al.* 2012). However, in this study, we have extended these observations by demonstrating simultaneous fluctuations in other skin bacteria. *Staph. epidermidis* is considered as the principal *Staphylococcus* species in the skin of controls (Iwase *et al.* 2010; Lai *et al.* 2010). The simultaneous prevalence of both *Staph. aureus* and *Staph. epidermidis* in Portuguese patients with AD was also observed in this study, which could provide a novel approach into the association between staphylococci as reported recently by Kong *et al.* (2012). These two species could possibly share a mutualistic or commensal association to improve frequent resistance to antimicrobial peptides (Peschel *et al.* 2001; Sieprawska-Lupa *et al.* 2004; Lai *et al.* 2007; Li *et al.* 2007) or improve binding to exposed extracellular matrix proteins in inflamed AD skin (Nilsson *et al.* 1998; McCrea *et al.* 2000; Cho *et al.* 2001; Williams *et al.* 2002). Otherwise, it might represent a compensatory mechanism of increasing *Staph. epidermidis*

numbers in an effort to control *Staph. aureus* (Iwase *et al.* 2010; Kong *et al.* 2012). To our knowledge, *Staph. hominis* species has never been reported as an important micro-organism in AD, but in our study, they constitute a relevant fraction of the isolates (27·8%), which possibly could reflect new approaches into the association between staphylococci species similar to those described above for the *Staph. epidermidis* species.

In a previously work (Tavaria *et al.* 2012), we characterized the staphylococcal community of 24 healthy Portuguese individuals by the same molecular-based approach resulting in 48 isolates included here as controls. Comparing these results with those obtained with patients with AD, a shift in the predominant strains and in the diversity of the skin microflora was seen, with nine species being detected in healthy individuals in contrast to only four species identified in AD. Therefore, the staphylococci identified in the present study may suggest a better adaptation of these strains to the AD environment. However, a cautious note should be made due to the lower number of isolates per individual, increasing interpersonal heterogeneity, which could in part influence the higher biodiversity of normal skin. However, Kong *et al.* (2012) also demonstrated in their study of the skin microbiome in children with AD, where increase in *Staph. aureus* accounted for reductions in skin microbial biodiversity. In fact, as a result of a skin barrier dysfunction and inflammation of the upper dermis, the skin of patients with AD exhibits a remarkable susceptibility to colonization with *Staph. aureus*.

The important role of colonization by *Staph. aureus* as an aggravating factor in AD is well established, as there is a significant correlation between the severity of the disease and *Staph. aureus* skin colonization (Bunikowski *et al.* 2000). Several strains of *Staph. aureus* present on atopic skin include a peculiar ability to produce exotoxins with SAg activity, which may play an important role in the natural course of atopic dermatitis.

Furthermore, the skin of patients with AD colonized with *Staph. aureus* harbouring SAg genes shows a significant increased severity of the disease as compared to patients colonized by *Staph. aureus* with no SAg genes (Nishijina *et al.* 1997; Roll *et al.* 2004; Nada *et al.* 2012). A multiplex PCR assay that allows for the rapid screening of the 19 genes that encodes SEs, SEls and TSST-1 in 21 *Staph. aureus* strains isolated from the skin of patients with AD is described in this study. As result, 16 of the 21 *Staph. aureus* strains (76%) showed the presence of toxin genes, while the remaining five strains (24%) did not harbour toxin genes. Previous studies have shown that more than 50–60% of *Staph. aureus* strains isolated from patients with AD are exotoxin-producing strains, which can secrete various exotoxins including SEA, SEB, SEC, SED and TSST-1 (Bunikowski *et al.* 2000; Chen *et al.* 2005; Silva *et al.* 2006; Schlievert *et al.* 2008; Nada *et al.* 2012).

More than one toxin gene was detected in all strains up to seven toxin genes detected simultaneously in one strain. The SElM and SElN (15 isolates, 71·4%) genes were the most frequently detected in our study, followed by SEG and SElO (14 isolates, 66·7%). All these genes were found together in the same strain with only one exception. Those genes are components of the enterotoxin gene cluster (egc), and the previously known egc type (SEG, SEI, SElM and SElN with SElO or SElU) (Hwang *et al.* 2007) was frequently distinguished in this study. Other studies also revealed a high prevalence of SAg genes associated with the egc locus in *Staph. aureus* isolates from patients with AD (Mempel *et al.* 2003; Bonness *et al.* 2008; Schlievert *et al.* 2008).

Although SEI and SEG are encoded on the same pathogenicity island, the egc cluster, frequently only SEG, was detected using the primer pairs described previously (Hwang *et al.* 2007). This effect may be caused by polymorphisms that were found in the egc cluster (Mempel *et al.* 2003). Moreover, the SEA (six isolates), SElL (seven isolates) and SElU (one isolate) genes were also detected, but in low frequencies. Nada *et al.* (2012) proposed that this diversity in toxin detection in atopics is associated with the severity of the disease and the site of the skin involved or sampled. This was also confirmed by Yagi *et al.* (2004) in a study where *Staph. aureus* strains were isolated from different skin areas in patients with AD, finding that 41% in the nonlesional area, 62% in the dry lesional area and 75% in the exudative lesional area were SAg-positive. Despite the proportion of toxin-producing strains and the frequency of certain toxins differing between studies (Breuer *et al.* 2000; Schlievert *et al.* 2008), generally, the SEB or SEC is the most predominant SAg gene detected in AD (Bunikowski *et al.* 2000; Zuel-Fakkar and El-Shokry 2010; Nada *et al.* 2012). In our samples, none of these two types of SAg genes were detected, which may be attributed to the limited number of isolates and/or individuals we studied. However, Arkwright *et al.* (2001) also demonstrated that the SEB was poorly detected (4%) in the skin of children with atopic dermatitis.

The relevance of evaluating the combination of virulence traits among staphylococci has been recently emphasized in both human and veterinary medicine (Zecconi *et al.* 2006). Therefore, a similar proportion of the isolates showed haemolytic activity in horse blood agar both in healthy and AD individuals.

In conclusion, this molecular-based approach successfully identified the staphylococcal microflora that was relatively specific to patients with AD. Therefore, identification and

fluctuation of certain micro-organisms and their association with certain disorders reveal the importance of these microbes in the course of human disease. The presence of SAg genes among coagulase-negative Staphylococcus and other SAgs should be further studied to obtain a more comprehensive profile in patients with AD. Extending our observations to a large number of patients, with a longitudinal study design, may be also of great help to fully understand the role of the skin staphylococci community in the exacerbations and pathogenesis of atopic dermatitis.

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Conflict of Interest

The authors declare that they have no conflict of interest

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II

Filaggrin Polymorphism Pro478Ser Is Associated With the Severity of Atopic Dermatitis and Colonization by *Staphylococcal aureus*

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Key words: Atopic dermatitis. Filaggrin mutation. p.Pro478Ser polymorphism. *Staphylococcus aureus*. Severity.

Palabras clave: Dermatitis atópica. Mutación de la filagrina. Polimorfismo P.Pro478Ser. *Staphylococcus aureus*. Gravedad.

Loss-of-function mutations in the filaggrin (*FLG*) gene are associated with increased severity of atopic dermatitis (AD) [1]. Common variants, such as p.Arg501Ter and 2282del4 may be present in up to 50% of Northern European AD patients and absent in Southern European patients [2]. rs11584340 (p.Pro478Ser) is a single-nucleotide polymorphism (SNP) of FLG that is located at codon 478. It is associated with skin barrier disruption, since the 478 serine residue may hinder the action of protease cleavage, thus affecting the rate of aggregation between FLG and keratin filaments [3,4]. Despite having a minor allele frequency of 0.34 worldwide [5], the polymorphism was found to be associated with an increased risk of AD (odds ratio, 1.87) [6,7]. Previous studies have reported that patients with null mutations in FLG have increased transepidermal water loss and increased skin pH, both of which facilitate bacterial growth [8]. However, it remains unknown how p.Pro478Ser affects predisposition to skin colonization by *Staphylococcus aureus* in AD patients.

We performed a cross-sectional analysis to evaluate the association between disease severity/colonization of the skin by *S aureus* and the polymorphism p.Pro478Ser and the null mutations in *FLG* (p.Arg501Terc and 2282del4).

Patients older than 12 years and diagnosed with AD according to the criteria of Hannifin and Rajka provided their written informed consent to participate in the study. In the case of minors, consent was given by the parents, caretakers, or guardians. The Ethics Committee of Porto University, Portugal approved the study. Participants with severe skin disease other than AD, secondary infection (bacteria, fungi, or viruses), or any major systemic disease were excluded. Sample size was calculated based on minimal clinically important differences in the SCORing Atopic Dermatitis (SCORAD) score [9], and post hoc statistical power was set at 95.6% (P=.05) based on the prevalence of FLG mutations in a previous study of Southern European AD patients [2]. We analyzed data from 73 patients (mean age, 30 [13] years; 61% female; 77% atopic) with AD for a mean (SD) of 16 years. Severity was classified based on the SCORAD score as mild (≤15), moderate (16-40), and severe (≥41). Genomic DNA was extracted from peripheral blood samples and analyzed using PCR and direct DNA sequencing for the presence of the 2 null mutations in *FLG* and the p.Pro478Ser polymorphism. The microbiological profile was assessed in the right and left elbow creases, left and right popliteal creases, and neck region (area, 25 cm^2). The number of colony forming units (CFU)/cm^2 of total staphylococci and *S aureus* was determined (Baird-Parker Agar [Lab M] for total staphylococci and Mannitol Salt Agar [Lab M] for *S aureus*). The serum biomarkers assessed were total IgE, eosinophil cationic protein, and specific IgE to a mixture of inhalant allergens (Phadiatop), *S aureus* enterotoxins (A, B, C, and TSST), and *Malassezia* species (ImmunoCap). The Mann-Whitney test or Fisher exact test was used as appropriate (IBM SPSS Statistics for Windows [Version 20.0], IBM Corp).

FLG mutations were present in 15% of patients (9 with p.Arg501Ter and 2 with c.2282del4) and p.Pro478Ser in 38% (3 homozygotes, 25 heterozygotes). p.Pro478Ser was in linkage disequilibrium with the null mutations, and 3 patients with the p.Arg501Ter mutation also had p.Pro478Ser. The presence of p.Pro478Ser was associated with more severe disease, as reflected by the higher SCORAD score and severity class as well as by increased use of oral corticosteroids (Table). Furthermore, significantly more extensive colonization of *S aureus* on 3 of the 5 sampled regions and a higher value of IgE to *S aureus* enterotoxin A were observed. Homozygosity for p.Pro478Ser was not an additional risk factor in this particular group of patients. There were no differences between patients with and without the *FLG* null mutations in terms of AD severity, inflammatory allergic markers, and colonization by *S aureus*.

The novel finding of this study is that, in contrast with the 2 *FLG* null mutations, p.Pro478Ser was significantly associated with more severe disease and greater skin colonization with *S aureus* in AD patients. The 478 serine residue can increase skin permeability, leading to greater skin penetration by bacteria and conferring susceptibility to AD [4]. In addition, the presence of an unrecognized functional mutation at or adjacent to *FLG*, which is in linkage disequilibrium with p.Pro478Ser, could increase the risk for AD [10]. Therefore, our findings indicate that this SNP may have clinically relevant implications with respect to increased bacterial colonization of skin and more severe disease in AD patients.

The limitations of this study are as follows. First, the absence of healthy controls restricts us to speculation on the role of this SNP in patients with AD. Nevertheless, our objective was to study the association between this SNP and bacterial load in patients and not the role of the SNP as a risk factor for AD, in which case it would have been mandatory to include healthy controls. Second, the prevalence of *FLG*

mutations in the Portuguese population as a whole and in AD patients in Portugal is unknown. However, the sample size calculations showed that 42 patients were needed to detect a significant difference in the SCORAD score, and we were able to include more patients to overcome the level of uncertainty regarding the prevalence of genetic mutations.

Importantly, this is the first study to show an association between the presence of p.Pro478Ser and severity of AD and bacterial load in European patients with long-term AD. Only 3 previous studies have investigated this SNP, although these were in Asian patients, suggesting that it confers susceptibility to AD, particularly in patients with high IgE levels [3,6,7]. The low prevalence of *FLG* null mutations in our study is consistent with the wide variation in this gene mutation across the globe and the lower prevalence in Southern European countries. The lack of an association with clinical, microbiological, and allergic parameters reinforces the fact that genetic markers other than *FLG* mutations should be studied.

In conclusion, genetic factors can affect the severity of AD and skin microbiota. Our study shows that the presence of p.Pro478Ser may be related to both increased disease severity and bacterial colonization in patients with long-term AD.

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Table. Characteristics of Patients With Atopic Dermatitis According to Filaggrin Genotype[a]

	FLG Null Mutations Mp.Arg501Ter or C.2282del4			FLG Polymorphism Pro478Ser		
	Yes, n=11	No, n=62	*P* Value	Yes, n=28	No, n=45	*P* Value
Age, y	32 (6.1)	29.6 (1,5)	.91[b]	34.1 (2.7)	27.3 (1.8)	.03[b,d]
Female sex, No. (%)	7 (63.6)	38 (61.5)	.22[c]	16 (57.1)	28 (62.2)	.42[c]
Disease duration, y	15.9 (10.5)	16.3 (10.4)	.23[b]	18.4 (2.3)	14.8(1.3)	.32[b]
SCORAD (0-103)	50.2 (30.9)	41.3 (22.6)	.72[b]	51.8 (4.2)	36.0(3.4)	<.01[b,d]
SCORAD severity, No. (%)						
Mild	2 (18.2)	5 (8.1)	.28[c]	2 (7.1)	5 (11.1)	.40[c]
Moderate	3 (27.3)	26 (41.9)	.81[c]	6 (21.4)	23 (51.1)	.02[c,d]
Severe	6 (54.5)	31 (50.0)	.52[c]	20 (71.4)	17 (37.8)	.01[c]
Oral corticosteroids, No. (%)	3 (27.3)	30 (48.4)	.22[c]	17 (60.7)	16 (35.6)	.03[c,d]
Atopic, No. (%)	6 (54.5)	50 (79)	.53[c]	22 (78.6)	34 (75.6)	.52[c]
Asthmatic, No. (%)	4 (36.4)	36 (58.1)	.64[c]	14 (50.0)	26 (57.8)	.31[c]
Median (IQR) total IgE, IU/mL,	2185 (71.4-5308)	4183 (97.3-3607.8)	.08[b]	6520 (113.6-7935.0)	2240 (88.6-1151.5)	.08[b]
Median Phadiatop, kU_A/L median (P_{25-75})	248.6 (4.5-565.9)	529.9 (0.54-441.0)	.12[b]	763 (9.6-1115.9)	315 (0.4-283.5)	.13[b]
ECP, µg/L	20.7 (14.9)	35.2 (29.1)	.56[b]	37.2 (34.2)	30.5 (21.1)	.52[b]
Specific IgE, kU_A/L						
Enterotoxin A	0.37 (0.2)	2.4 (1.3)	.79[b]	4.5 (13.9)	0.5 (0.9)	.05[b,d]
Enterotoxin B	0.6 (0.3)	1.5 (0.5)	.42[b]	2.4 (5.1)	0.6 (1.3)	.23[b]
Enterotoxin C	1.3 (0.5)	2.2 (0.5)	.38[b]	2.7 (3.5)	1.6 (3.1)	.06[b]
Enterotoxin TSST	0.5 (0.2)	1.4 (0.6)	.52[b]	2.4 (6.7)	0.4 (0.8)	.08[b]
Malassezia species	6.2 (5.8)	4.2 (1.1)	.78[b]	7.2 (13.4)	3.3 (8.7)	.23[b]
Staphylococcus aureus, CFU/cm^2						
Right arm	9471.1	78 152.7	.48[b]	178 083.3	8002.3	.01[b,d]
Left arm	158 909.9	70 271.9	.58[b]	142 859.2	48 310.3	.92[b]
Right leg	23 454.4	39 728.2	.91[b]	89 778.9	8 386.7	.04[b,d]
Left leg	162 754.4	359 865.8	.96[b]	759 552.7	95 528.5	.02[b,d]
Neck	8 994.9	30 732.6	.74[b]	48 538.3	16 244.8	.80[b]

Abbreviation: ECP, eosinophil cationic protein; SCORAD, SCORing Atopic Dermatitis.
[a]Results are presented as mean (SD) unless stated otherwise.
[b]Mann-Whitney test.
[c]Fisher exact test.
[d]Statistically significant.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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III

Personality traits may influence atopic dermatitis severity in adult patients: pilot study

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Atopic dermatitis (AD) is a relapsing inflammatory skin disease that when persisting into adulthood tends to be more severe, with great impact in quality of life of patients and their families[1]. It has a complex pathogenesis including skin barrier impairment, innate and Th2 driven immunological deregulation[1]. Psychological factors may play a pivotal role in AD manifestations as distress symptons potentiate the release of pruritogenic neuromediators and trigger skin inflammation[2]. Personality traits, defined as the way individuals think, feel and behave modulate the way patients minimize or tolerate stress or conflicts (coping strategies)[3]. Previous meta-analyses in other chronic diseases had linked higher scores on personality traits as extraversion, conscientiousness and openness to more engagement coping, in contrast with higher scorers on neuroticism that show a tendency for inadequate disease managing strategies[4]. Concerning AD it is not known if personality traits may influence AD severity on a real life setting.

In this pilot study, we assessed the relation between the 5-main domains of personality traits and objective AD severity in adult patients with long-term disease.

In this cross-sectional analysis, subjects older than 16 years with a medical diagnosis of AD according to the criteria of Hannifin and Rajka attending hospital visits were invited to participate. AD severity was assessed trough SCORAD index (0-103), personality traits through the short version of the NEO Personality Inventory (NEO-PI-R) [5] already validated in the Portuguese population[6]. This is a 60-item multiple choice questionnaire that evaluates the 5 main dimensions of personality: neuroticism (measure for emotional stability), openness (the predisposition to new experiences), extraversion (the main energy focus being held in- or outwards), agreeableness (the ability to deal with others) and conscientiousness (the sense of

right and wrong towards own behaviour). The local ethics committee approved the study and informed consent was obtained from all. Participants with severe skin disease other than atopic dermatitis, secondary infection with bacteria, fungi, virus or any major systemic disease were excluded. Sample size calculations were performed to determine the number of participants needed to detect effect sizes based on minimal clinically important differences in the SCORAD index: 42 patients were needed to detect a difference with a two-sided 0.05 significance level and a probability of 81% if the true difference in SCORAD between groups was 8.7 units. From the 78 patients invited, 46 agreed to participate during hospital visits, and two were excluded because of significant comorbidities, diabetes mellitus type 1 and multiple sclerosis. Data from 44 patients (30±13 years, 61% female, 77% atopic) with AD for 16±10 years were analysed. Eleven (25%) had mild, 18 (41%) moderate, and 15 (34%) severe AD , SCORAD (mean±sd) was 44.9±27.3. One-way ANOVA test was applied; when significant differences were found a post-Hoc Bonferroni correction was performed.

We found that subjects scoring “high” on conscientiousness had less severe disease than those scoring ‘normal’: mean (95% CI) SCORAD of 31.17 (19.58 to 42.58) vs. 56.16 (42.73 to 68.67); p =.039, respectively. No further differences were observed concerning neuroticism (p =.960), extraversion (p =.065), openness (p =.722) or agreeableness (p =.186) traits (Table).

The fact that personality traits may influence atopic dermatitis severity is an important finding of our study. In contrast with results from a previous experimental setting [7], we found that higher conscientiousness was associated with less severe disease. Conscientiousness is associated with being methodical, hardworking,

efficient and organized, focused on solving tasks effectively and results-oriented[3].It has been also previously linked to a consistent protective effect, predicting lower risk for internalizing problems. [4]. We hypothesize that this personality trait may had enhanced treatment compliance and diminish the impact of stressful stimulus. We also observed that patients scoring low in extraversion and high in neuroticism tended to have higher SCORAD mean values. This may be explained by emotional instability, dominated by vulnerability to experiences of anxiety and general distress.

As for study limitations, we refer to the inability to assess the causal directionality of the associations given its cross sectional design. Second, the limited sample size. Nevertheless, our study had a similar number of included patients as the two previous studies addressing this subject [7, 8] and we managed to include the adequate number of patients according to sample size calculation.

Our study has some important strengths: it was carried out in an outpatient setting with validated clinical outcomes as previously suggested by other authors[7]; we had applied the same psychological questionnaires that had been used in a recent European Multicentre survey[9] and that were previously validated in the Portuguese population[10] facilitating future comparisons. Furthermore, it is the first study to explore the relation between personality traits and AD severity in a clinical setting.

We conclude that personality traits may influence AD severity in adult patients with long term disease. Longitudinal studies addressing the role of personality in attaining AD control are needed to draw definite conclusions. Psychological assessment and training of adaptive coping strategies enhancing self-control may benefit patients with chronic atopic dermatitis.

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Table . Personality traits and severity of atopic dermatitis

			SCORAD		
Personality traits	Categories	N(%) patients	Mean	95% CI	p-value*
Neuroticism	Low	9 (20)	47.21	22.23 to 71.66	0.960
	Normal	21 (48)	45.22	32.54 to 57.27	
	High	14 (32)	44.23	28.39 to 58.61	
Extraversion	Low	2 (5)	83.12	-190.68 to 355.68	0.065
	Normal	18 (40)	37.14	24.95 to 49.72	
	High	24 (55)	47.16	36.05 to 58.70	
Openness	Low	3 (7)	42.21	-10.79 to 95.46	0.722
	Normal	25 (57)	48.43	35.03 to 60.57	
	High	16 (36)	41.32	28.51 to 52.79	
Agreeableness	Low	13 (30)	55.13	38.07 to 72.55	0.186
	Normal	20 (46)	38.31	27.65 to 48.93	
	High	12 (24)	48.12	20.96 to 75.04	
Conscientiousness	Low	12 (27)	41.13	22.43 to 58.74	**0.035**
	Normal	20 (45)	56.16	42.73 to 68.67	
	High	12 (28)	31.17	19.58 to 42.58	

SCORAD: Score index of atopic dermatitis severity,:CI-confidence interval *one-way ANOVA test; patients scoring very low and low or very high and high were grouped into low and high respectively

IV

ORIGINAL ARTICLE SKIN AND EYE DISEASE

Functional textiles for atopic dermatitis: a systematic review and meta-analysis

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atopic dermatitis; systematic review; functional textiles; immunomodulation

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Abstract

Atopic dermatitis (AD) is a relapsing inflammatory skin disease with a considerable social and economic burden. Functional textiles may have antimicrobial and antipruritic properties and have been used as complementary treatment in AD. We aimed to assess their effectiveness and safety in this setting. We carried out a systematic review of three large biomedical databases. GRADE approach was used to rate the levels of evidence and grade of recommendation. Meta-analyses of comparable studies were carried out. Thirteen studies (eight randomized controlled trials and five observational studies) met the eligibility criteria. Interventions were limited to silk (six studies), silver-coated cotton (five studies), borage oil, and ethylene vinyl alcohol (EVOH) fiber (one study each). Silver textiles were associated with improvement in SCORAD (2 of 4), fewer symptoms, a lower need for rescue medication (1 of 2), no difference in quality of life, decreased *Staphyloccosus aureus* colonization (2 of 3), and improvement of trans-epidermal water loss (1 of 2), with no safety concerns. Silk textile use was associated with improvement in SCORAD and symptoms (2 of 4), with no differences in quality of life or need for rescue medication. With borage oil use only skin erythema showed improvement, and with EVOH fiber, an improvement in eczema severity was reported. Recommendation for the use of functional textiles in AD treatment is weak, supported by low quality of evidence regarding effectiveness in AD symptoms and severity, with no evidence of hazardous consequences with their use. More studies with better methodology and longer follow-up are needed.

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease with a considerable social and economic burden; it has an estimated prevalence of up to 20% in children and 2% in adults (1, 2). Its pathophysiology is complex and involves skin barrier defects and immunologic deregulation in genetically predisposed individuals (3–5). The skin of patients with AD is particularly susceptible to infection by different microorganisms. It is frequently colonized with *Staphylococcus* species capable of producing several virulence factors that contribute to the perpetuation of skin inflammation, even in normal-appearing skin (6). Disease management thus demands an integrated approach, aimed not only at diminishing pruritus, controlling skin inflammation, and ensuring skin hydration but also at regulating the skin microbiome (7, 8).

Textiles are considered an important part of AD management, and fabrics such as cotton and silk garments tend to reduce scratching and aid emollient absorption (9). With the development of nanotechnology, intelligent, or functional, textiles, which are designed to have beneficial effects on human health, have emerged (10). Such textiles have been used as adjuvants and antiseptic dressings in burns and wound healing with promising results (11, 12). In immunologically mediated skin diseases, and AD in particular, the focus has been to improve itch sensation, severity of lesions, and skin colonization by *S. aureus*.

Most of the studies of functional textiles in AD have investigated the use of specially treated long-sleeved shirts and pants in close contact with the skin. Cotton textiles can be functionalized with antiseptic silver salts (13, 14) or borage oil, which supplies fatty unsaturated acids to the skin barrier (15). Silk coated with specific antimicrobial chemical compounds and smooth ethylene vinyl alcohol (EVOH) fibers are also used to diminish physical stimuli applied to the skin (16). Nonetheless, contact between bioactive compounds in functional

textiles and a disrupted skin barrier raises safety concerns, although the few studies addressing the potential risks of sensitization, disturbance of the ecology of the skin, and toxic side effects have shown functional textiles to be safe and usable (17).

Although functional textiles may be a promising area in skin disease management, their role in AD has not yet been established. The aim of this study was to systematically review the efficacy and safety of these textiles in AD.

Methods

We selected published reports of randomized controlled trials (RCTs) and observational and case studies (with a cohort or case–control design) that compared or assessed the effects of functional textiles in patients of any age with a clinical diagnosis of atopic dermatitis; no restrictions were placed on disease severity or previous or current treatment.

The primary outcome was defined as changes in overall eczema severity, measured by the SCORing Atopic Dermatitis (SCORAD) index and other scales for evaluating AD severity (18). Secondary outcomes included changes in symptoms, quality of life, need for rescue medication, microbiologic skin flora composition, epidermal skin physiology, and safety.

Search strategy

In July 2012, electronic searches were undertaken in three large biomedical databases: the Cochrane Central Register of Controlled Trials, Scopus, and Medline. We used the following keywords (first group): 'atopic eczema dermatitis syndrome', 'atopic dermatitis', 'atopic eczema', coupled with (second group) 'textiles', 'fabrics', 'garments', 'clothes', and 'dressings'. *A priori* inclusion criteria limited retrieved articles to those assessing the use of textiles in individuals with AD. Subsequently, each study was evaluated to determine whether it met the entry criteria for the review. Hand searches of the reference lists of all pertinent reviews were performed and potentially relevant studies identified. Abstracts from relevant conferences were also searched. After the electronic literature searches, using the title, abstract, or both, two authors independently selected articles for full-text scrutiny. The authors agreed on a set of articles, which were retrieved and assessed to determine compliance with the entry criteria. Information regarding the following characteristics was extracted from each study: design (description of randomization, blinding, number of study centers, and number of study withdrawals); participants (sample size), mean age, age range of the population; intervention (type and study duration); and outcomes (type of analysis and outcomes analyzed). The results of comparable studies for a specific outcome were pooled using a random effects meta-analysis (19).

Grading system

Evidence was graded based on an analysis of outcome measures. The overall quality of evidence is presented using the GRADE approach recommended by the Cochrane Handbook for Systematic Reviews of Interventions (19). That is, for each specific outcome, five factors were scrutinized: (i) limitations of the study design or the potential for bias across all studies accordingly to the measure of a particular outcome, (ii) consistency of results, (iii) directness (generalizability), (iv) precision (sufficient data), and (v) the potential for publication bias. The overall quality was considered to be high if multiple RCTs with a low risk of bias provided consistent, generalizable results for the outcome. The quality of evidence was downgraded by one level if one of the factors described above was not met. Likewise, if two or three factors were not met, the level of evidence was downgraded by two or three levels, respectively. Thus, the GRADE approach resulted in four levels of quality of evidence: high, moderate, low, and very low. When a given outcome was measured by only one study, data were considered to be 'sparse', and subsequently, the evidence was labeled as 'low quality'. The systematic approach suggested by the GRADE working group was followed using the GRADE profiler software (version 3.2 for Windows. Jan Brozek, Andrew Oxman, Holger Schünemann, 2008) (20–24).

Quality of evidence classification is needed to ascertain whether an estimate of the effect is adequate to support a particular recommendation for the clinician. Strength of recommendation was performed according to the quality of the supporting evidence and classified as strong or weak for the use of functional textiles, through the balance of desirable/undesirable outcomes (20–24).

Results

Thirteen studies met the eligibility criteria and were included in our review. Fig. 1 shows the flow chart of the study selection strategy, and Table 1 shows the studies included. One study, an expert's bibliographic review, was excluded because did not met the inclusion criteria (25). Table 2 includes the classification of functional textiles according to their active compound.

The studies included participants aged between 4 months and 70 yr, with no restriction in disease severity. The interventions included silver (13, 14, 17, 26, 27), silk (28–33), borage oil (15), and EVOH fiber (16) used for a period of 1–12 wk. RCTs addressed silk textiles in two studies (28, 32), silver-coated textiles in 4 (13, 17, 26, 27), and borage oil (15) and EVOH fiber (16) in one study each. The case–control studies analyzed silk fabric (30, 31) and silver-coated textile (14). Silk textiles were also examined in one side-by-side comparison study (29) and one uncontrolled study (33). Silver-coated fabrics were studied in both children and adults in all cases (13, 14, 17, 26, 27). Silk, by contrast, was studied mostly in children (28–31), and borage oil (11) and EVOH fiber (12) were studied in children only. Control textiles included cotton (for studies of silver, borage oil, and EVOH fiber) and regular silk for studies of silk with AEGIS antibacterial treatment (28, 30–32). All the studies addressed eczema severity, measured by SCORAD (13–17, 26–28, 30, 32) and the Eczema Area and Severity Index (EASI) (29, 33). The skin microbiome was analyzed in studies of silver (14, 17, 26) and silk (31), while skin physiology was studied in those of silver and borage oil (15, 26, 27). Safety was assessed in studies of silver (13, 17, 26) and silk (29) textiles. Considering the reported outcomes, all the studies

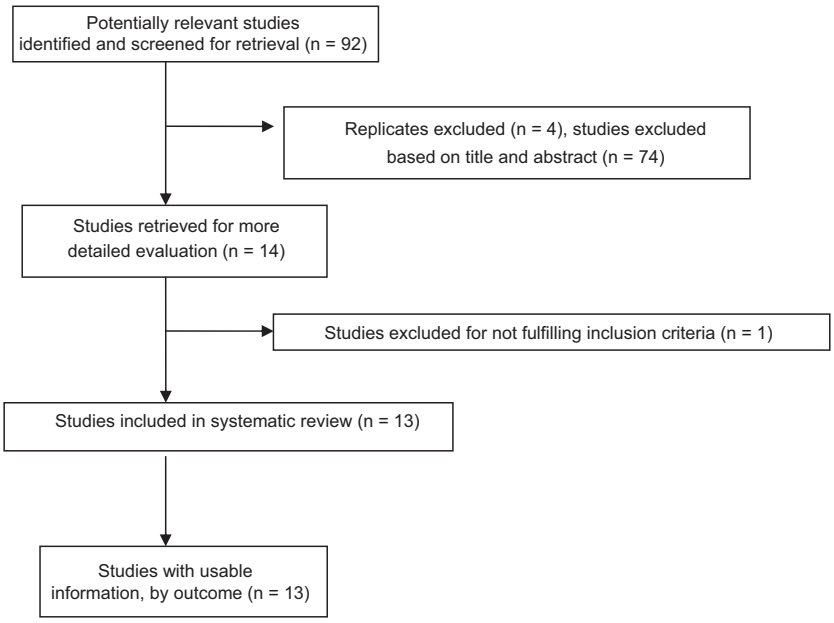

Figure 1 Flow-chart of included studies.

were deemed to have a low or very low quality of evidence (Table 3, Grade Evidence Profile and Table S1).

AD severity (SCORAD)

SCORing Atopic Dermatitis was used in 10 studies (13–17, 26–28, 30, 32), involving all kinds of interventions. Compared with placebo, a significant improvement in disease severity was observed for silver in two studies (13, 27) and for silk, also in two studies (28, 32). In the remaining studies, there was a reduction in disease severity, but no comparisons were made with placebo.

Meta-analysis was possible in two RCTs of silver-coated fabrics reporting a reduction in eczema severity (mean difference −12.66 [−21.26; −4.07], $I^2 > 60\%$) (13, 17) (Fig. 2).

AD severity (EASI)

Two studies analyzing silk used the EASI to evaluate AD severity. Senti et al., using a side-by-side comparison method, showed a significant decrease in severity, but they did not detect any differences between the side of the body in contact with the treated silk fabric and the other side (29). Kurtz et al. (33), in an uncontrolled study, reported a decrease in EASI following the use of a silklike-bedding fabric.

Symptoms

Five studies, using silver (13, 17), silk (28, 32), and borage oil (15), reported AD symptoms of pruritus and sleep loss as separate outcomes. In the silver group, no significant differences were found in the trial by Gauger et al. (13) for pruritus and sleep loss; Juenger et al. (17), by contrast, showed a significant reduction in symptoms in individuals who used silver textile, but they did not perform a comparison with placebo. In studies examining silk, a significant improvement in symptoms was seen in the active group; these studies were included in a meta-analysis due to their homogeneity (mean difference −1.74 [−2.19; −1.30], $I^2 = 0\%$) (28, 32) (Fig. 3). The trial of cotton undershirts coated with borage oil also reported a reduction in symptoms in the active group, but there was no comparison with placebo (15).

Quality of life

Quality of life in patients with AD was assessed using different tools. Gauger et al. (13), using the German Instrument for Assessment of Quality of Life in Skin Diseases (DIELH), showed an overall improvement in quality of life among patients who wore silver-coated garments, but they did not detect any significant differences with patients who wore untreated cotton garments. Kurtz et al. (33), using a study-specific quality of life index, assessed every 2 wk up to 8 wk, saw a progressive improvement in quality of life in patients who used silklike bedding, but there was no comparison with controls.

Rescue medication

The use of rescue medication (topical corticosteroids) was addressed only in studies evaluating silver textiles. Juenger et al. (17), using data from the first 2 wk of the trial, analyzed the use of prednicarbate ointment (measured in grams) as rescue medication in three groups (those who used silver textile, those who used silver-free textile, and those who used prednicarbate ointment regularly), and found that the quantity

Table 1 Studies included in the systematic review

References	Study design and subjects	Intervention	Outcome	Results
Gauger et al. (14)	Case–control; 15 subjects, aged 3–55 yr	Silver-coated tubular sleeves vs. cotton for 7 days, and 7 days follow-up	AD severity	Reduction in SCORAD score in silver-coated textile (no comparison with placebo)
			Microbiome	Significantly lower *Staphylococcus aureus* colonization
Ricci et al. (30)	Case–control; 46 children aged 4 months–10 yr	Silk undershirts, leggings, tubular sleeves on arms, and legs vs. cotton for 7 days	AD severity	Significant decrease in SCORAD index (reduction in mean local score ($p = 0.001$) in active group. No comparison with placebo
Juenger et al. (17)	RCT; 30 subjects, aged 4–70 yr	Undershirts and pants for 2 wk: silver vs. cotton vs. prednicarbate ointment, followed by silver textile in all groups	AD severity	Improvement in SCORAD index in the first 2 wk: from 74.60 to 29.95 in the silver group ($p = 0.005$) and from 57.80 to 24.00 in the steroid group ($p = 0.009$). No comparison with placebo
			Symptoms	Reduction in pruritus severity in the silver group ($p = 0.031$)
			Rescue medication	Similar to regular steroid group, more than in placebo group
			Microbiome	Significant reduction ($p = 0.003$).
			Safety	No adverse event
Gauger et al. (13)	Double-blind RCT; 57 subjects, median age 17.7 yr	Silver-coated tubular long-sleeves and long-legged pants for 2 wk	AD severity	Reduction in SCORAD index: 27.4% in silver group and 16.3% in placebo ($p < 0.001$)
			Symptoms	Improvement in pruritus and sleep, nonsignificant differences between groups
			Quality of life	Improvement in 18.9% from baseline compared with 17.1% in placebo; no significant differences between groups
			Rescue medication	16% less topical steroids in active group versus placebo
			Safety	No side effects
Senti et al. (29)	Side-by-side comparison study; 15 children, 1–5 yr	Silk vs. cotton with topical steroids	AD severity	No difference between groups
			Symptoms	No difference between groups
			Safety	One flare-up in active group
Ricci et al. (31)	Case–control, 16 children, aged 2–8 yr	Tubular sleeves made of silk vs. antimicrobial silk for 7 days	AD severity	Reduction in local SCORAD index in active and placebo groups ($p = 0.019$ and $p = 0.02$) No comparison between groups
			Microbiome	No significant reduction in *S. aureus* colonization in both groups
Khanehara et al. (15)	Double-blind RCT in 32 children, aged 1–10 yr	Undershirts coated with borage oil vs. cotton	AD severity	Reduction in erythema ($p = 0.033$)
			Symptoms	Reduction in pruritus ($p = 0.033$)
			Skin physiology	Significant decrease in TEWL ($p = 0.0480$), no differences with placebo group
Yokoyama et al. (16)	Double-blind RCT, 21 children aged 3–9 yr	EVOH fiber underwear for 4 wk	AD severity	Improvement in SCORAD index in active group only ($p = 0.001$). Objective SCORAD improvement in both groups No comparison between groups
Koller et al. (28)	RCT, 22 children, aged 5–12 yr	Tubular sleeves made of silk vs. antimicrobial silk for	AD severity	Reduced severity in active group in the first 2 wk ($p < 0.05$) but not

Table 1 (Continued)

References	Study design and subjects	Intervention	Outcome	Results
		2 wk, followed by cotton vs. antimicrobial silk for 10 wk		significant when compared to simple silk; significant differences at 4, 8 and 12 wk ($p < 0.001$)
			Symptoms	No difference in symptoms in the first 2 wk, significant differences at 4, 8, and 12 wk ($p < 0.001$)
Stinco et al. (32)	Double-blind RCT, 30 patients aged 3–31 yr	Tubular sleeves with silk coated with antimicrobial compound vs. silk	AD severity	SCORAD reduction significantly higher in active group (mean 10.05 ± 9.22, $p < 0.0001$)
			Symptoms	Decrease in pruritus in both groups, mean value of pruritus between groups favors active group
Kurtz et al. (33)	Uncontrolled study, 37 patients, aged <1 –69 yr	Silklike-bedding fabrics	AD severity	Significant decrease in AD area and severity index
			Symptoms	Significant decrease in itch score
			Quality of life	Increase in study-specific quality of life score
Fhur et al. (26)	Single-blind RCT; 37 subjects aged 12–60 yr	Silver-loaded T-shirts for 12 wk	Microbiome	Significant reduction in *S. aureus* colonization
			Skin physiology	Reduction in TEWL ($p = 0.0171$) in mildly involved skin in the silver group; nonsignificant improvement in severely involved areas
			Safety	No adverse events
Park et al. (27)	RCT single–blinded study; 14 subjects aged 6–35 yr	Silver vs. cotton T-shirts and leggings, side-by-side comparison for 4 wk	AD severity	Reduction in SCORAD index in active group
			Skin physiology	Reduction in TEWL ($p = 0.008$, 95% CI 1.1–6.71)

AD, atopic dermatitis; CI, confidence interval; EVOH, ethylene vinyl alcohol; RCT, randomized clinical trial; SCORAD, SCORing for atopic dermatitis; TEWL, transepidermal water loss.

Table 2 Classification of functional textiles according to active compounds

Functional textile	Textile composition	Type of fabric	References
Silver	Silver-loaded cellulose fabric with incorporated seaweed	Long-sleeved shirts and leggings	(26, 27)
	Silver-coated nylon fibers	Long-sleeved shirts and leggings	(17)
	Silver coated nylon fibers and polyamide	Long-arm undershirts and pants for adults, whole-body clothes for children	(13, 14)
Borage oil	Borage oil chemically bonded to cotton fibers	Undershirts	(15)
Ethylene vinyl alcohol fiber	Alternately arranged hydrophilic and hydrophobic nanoscale segments	Underwear	(16)
Silk	Sericin-free silk treated with AEGIS/AEM 5772/5	Tubular sleeves	(28, 31, 32)
		Whole-body romper suites, long-sleeved T shirts, panty hoses	(29)
	Microair Sericin-free silk treated with AEGIS/AEM 5772/5	Body suits, rompers, leggings, tubular bands, gloves, waist bands	(30)
	Silklike 50% polyester and 50% nylon	Bedsheets	(33)

of rescue medication used by patients in the silver group was similar to that used by the regular steroid group and higher than that used in the silver-free group. In the study by Gauger et al. (13), the percentage of patients who needed topical steroids was 16% lower in the group that wore silver-coated garments than in the group that wore cotton garments.

Table 3 Summary of findings. Functional textiles for atopic dermatitis

Patient or population: patients with atopic dermatitis; intervention: functional textiles					
	Illustrative comparative risks* (95% CI)				
Outcomes	Assumed risk	Corresponding risk	No. of participants (studies)	Quality of the evidence (GRADE)	Comments
Severity – SCORAD SCORAD and adaptations[1] Scale: 0–103	The mean SCORAD score in the control groups was 30.4 points[2]	The mean SCORAD score in the intervention groups was 12.7 lower (4.07–21.26 lower)	77 (2)	⊕⊕⊖⊖ low[3]	Data presented are only from the studies included in the meta-analysis
Other severity scales [4] EASI Follow-up: mean 4 wk	Not estimable	Not estimable	67 (2)	⊕⊖⊖⊖ very low[3,5]	
Patient-rated symptoms Visual scale analogic (0–10) Scale: 0–10	The mean patient-rated symptom score in the control groups was 5.55 points	The mean patient-rated symptom score in the intervention groups was 1.74 lower (2.19–1.3 lower)	104 (2[6])	⊕⊕⊖⊖ low[3,5,6]	Data presented are only from the studies included in the meta-analysis
Quality of life Quality of life questionnaire Scale from 0 to 110 Follow-up: mean 8 wk	Not estimable	Not estimable	94 (2[5,7])	⊕⊖⊖⊖ very low [5, 7,	
Need for rescue treatment Weight (in grams) of moderately potent corticosteroid cream used Scale: 0–200 Follow-up: 2 wk	Not estimable	Not estimable	87 (2[8])	⊕⊕⊖⊖ low[3,5,8]	
Skin microbiome Reduction in number of colony-forming units of *Staphylococcus aureus* Scale: 0–10 Follow-up: 8 wk[9]	Not estimable	Not estimable	122 (4)	⊕⊖⊖⊖ very low[3,5]	
Skin physiology Transepidermal water loss. Scale from 0 to 15 Follow-up: 8 wk	Not estimable	Not estimable	93 (3[10])	⊕⊕⊖⊖ low[3,11]	

Table 3 (Continued)

Patient or population: patients with atopic dermatitis; intervention: functional textiles					
	Illustrative comparative risks* (95% CI)				
Outcomes	Assumed risk	Corresponding risk	No. of participants (studies)	Quality of the evidence (GRADE)	Comments
Safety Urine and serum silver levels Follow-up: 8 wk	Not estimable	Not estimable	65 (2[12])	⊕⊖⊖⊖ very low[3,13]	
Dropout due to eczema flare-up Timing of exposure: 2 wk	Not estimable	Not estimable	15 (1)	⊕⊖⊖⊖ very low[14]	

*The basis for the assumed risk (e.g., the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).

CI: confidence interval; OR: odds ratio; SCORAD, SCORing Atopic Dermatitis (SCORAD) index.

GRADE Working Group grades of evidence.

High quality: Further research is very unlikely to change our confidence in the estimate of effect.

Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.

Low quality: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.

Very low quality: We are very uncertain about the estimate.

[1]SCORAD was evaluated using four variations: mean total SCORAD score (13, 17, 30), objective SCORAD index (30, 32), local SCORAD index (14, 28, 31), and modified local SCORAD index (15, 27).

[2]Final SCORAD score.

[3]Small sample.

[4]Evaluated in studies with a different methodology: Senti et al. (29) (side-by-side comparison study) and Kurtz et al. (33) (uncontrolled study).

[5]Lack of allocation concealment.

[6]Patients also served as controls in both studies.

[7]Different study designs linked together: Gauger et al. (13) (randomized controlled trial) and Kurtz et al. (33) (silklike-bedding, no control group).

[8]Data reported only by Juenger et al. (17). The use of rescue medication was compared between three groups in the first 2 wk of the trial (135 g of corticosteroid ointment per participant in the silver-textile group, 13 g in the silver-free textile group, and 145 g in the topical corticosteroid group. Gauger et al. (13) only reported the percentage of patients using topical steroids as rescue medication (84.4% in placebo group versus 68.6% in the silver group).

[9]All interventions lasted 1–2 wk except in the Fluhr et al. (26) study, in which they lasted 8 wk.

[10]Two randomized controlled trials (26, 27) assessing silver textile and one assessing borage oil (15). Park et al.(27) performed a side-by-side comparison study.

[11]The silver textile trials (26, 27) were single-blinded.

[12]One randomized control trial (26) tested serum silver measurements and a phase II randomized trial (17) measured urinary silver levels (only the first 2 wk of the intervention were considered).

[13]Single-blinded study, randomization methods not described. Outcome divided according to mild and severe atopic dermatitis, not detailed in methods (26).

[14]Side-by-side intervention study.

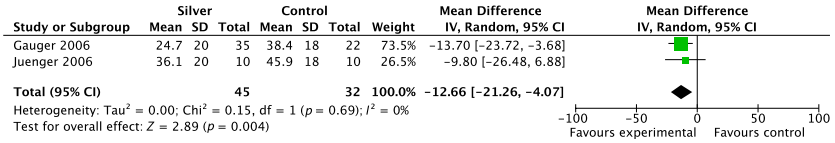

Figure 2 Metanalysis of SCORAD results (silver functional textiles versus placebo).

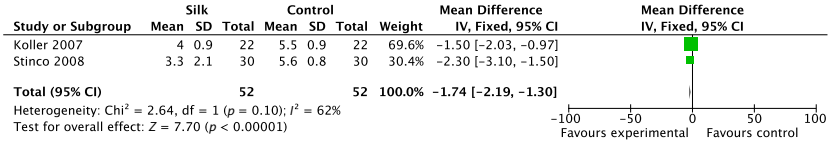

Figure 3 Metanalysis of atopic dermatitis symptons results (silk functional textiles versus placebo).

Skin microbiome

The effect of interventions on the skin microbiome was evaluated in terms of *S. aureus* colonization (mean number of colony-forming units [CFUs] per cm^2). Of the three studies analyzing silver textiles (14, 17, 26), the two RCTs (17, 26) showed a significant reduction in *S. aureus* colonization. In a case–control study of a silk fabric coated with AEGIS, a non-significant reduction in CFUs was seen in both cases and controls (31).

Skin physiology

Skin physiology was assessed by transepidermal water loss (TEWL) in three studies: two involving silver (26, 27) and one involving borage oil (15). In a side-by-side comparison study, compared with placebo, a significant decrease in TEWL was detected after 4 wk in patients who wore a silver-loaded fiber (26). In the other study of silver, similar results were obtained for mildly involved skin, but not for skin with more severe disease (27). In the borage oil study, TEWL decreased in the study and control groups, but the differences were not significant (15).

Safety

The systemic absorption of silver through the skin in patients who wore fabric impregnated with silver was evaluated by urine and serum silver measurements in two studies (17, 26), with no persistent increases detected. In a study of an antimicrobial silk fabric by Senti et al. (29), one of the patients dropped out at day 4 due to a flare in both treated and untreated skin areas.

Discussion

This systematic review found that the use of functional textiles in atopic dermatitis is safe and associated with a slight improvement in disease severity, symptoms, and quality of life. However, any recommendations for the use of these textiles as part of standard AD management are hampered by the low quality of supporting evidence. Different textile components are associated with different effects. Silver-coated cotton, for example, seems to be more effective in decreasing lesion severity, while silk fabrics appear to be more likely to alleviate pruritus and symptoms.

The evidence for the effectiveness of functional textiles in AD was qualified using the GRADE approach. In addition to an overall lack of evidence supporting the use of functional textiles in AD, the quality of evidence in the studies included in our review was either low or very low, mainly because they were non-randomized, non-controlled studies, which furthermore were underpowered to detect treatment effects due to small sample sizes. Short follow-up might also have reduced the ability to see true effects, possibly explaining why some studies did not detect differences between placebo and intervention groups. The use of different textiles, with different active compounds and therefore different physical and antimicrobial properties, prevented direct comparisons between studies. Accordingly, we only performed a meta-analysis of studies that evaluated the same interventions and outcomes. The limitations of this review are explained by the limitations of the studies included.

Atopic dermatitis is a complex disease that requires a multidimensional treatment approach (34). Control of environmental factors (35) and dietary intervention (36) have been proposed as the ultimate focus on atopic dermatitis management endorsing tolerance, prevention, and promotion of health attitudes instead of prompt medical treatment (37). Non-pharmacological strategies, as functional textiles, have been studied and represent an interesting therapeutic option for patients with AD (34, 38).

All the studies analyzed in this review that addressed eczema severity reported some benefits from using functional textiles, but the majority did not compare results with those from a control group. Due to differences in study design, interventions, and outcome measures, we were only able to pool data on SCORAD in two studies (13, 17), both of which analyzed silver-coated textiles. The meta-analysis showed a trend in favor of the use of these textiles.

Silver seems to exert its effect on eczema severity through its antimicrobial properties (39), diminishing colonization by *S. aureus* and consequently attenuating inflammation and consequent exacerbation of lesions. Nevertheless, definitive conclusions cannot be drawn, as we analyzed only two studies, with different designs and small sample sizes.

Silk textiles may affect overall disease status by improving comfort and reducing itch sensation. Almost all the studies of silk analyzed in this review used specific types of silk fabrics made of transpiring and slightly elastic woven silk, free of sericin (a protein assumed to be irritant to the skin), and impregnated with AEGIS, an antibacterial compound (28–32). The exception was the study by Kurtz et al. (33), which did not state which antimicrobial was used. Silk did not have a significant effect on *S. aureus* colonization, although this was analyzed in just one study (29). The use of silver textile, however, was significantly associated with a reduction in *S. aureus* colonization; the difference in effects may possibly be due to different mechanisms of action. (39) The use of EVOH fiber in AD is intended to reduce pruritus, as fabrics treated with EVOH have a smooth texture. However, in our review, the single study that analyzed EVOH fiber reported an improvement only in erythema. Borage oil has been previously used in AD to restore skin barrier lipids as an oral supplement, with conflicting results (40, 41). The lack of comparison with placebo in the study analyzing borage oil–coated textiles in our review (15) made it impossible to draw any definitive conclusions on effectiveness.

Functional textiles used in AD are designed not only to reduce disease severity, but also to alleviate symptoms. In most cases, the aim is to improve pruritus and sleep loss, two of the most distressing features of AD. Most of the studies we reviewed reported improvements in pruritus and sleep disturbance following the use of specially treated fabrics, but in half of the studies, no between-group comparisons were made. The use of silver-impregnated cotton fabric with an antimicrobial effect may contribute to the relief of symptoms. The two studies that analyzed silk reported a significant decrease in symptoms, and the meta-analysis of pooled data suggested that this fabric might be effective in improving the symptoms of AD. However, due to the small number of studies and small sample sizes, a definitive conclusion cannot be drawn.

A reduction in symptoms and colonization by *S. aureus* may also have an impact on quality of life. Nevertheless, the different tools used to measure this outcome—and the different study designs—prevent any conclusions from being made. The need for rescue medication was addressed in two studies (13, 17), but the results are not comparable as different outcomes were used (quantity of medication used and percentage of participants requiring medication).

The impact of the use of functional textiles on the skin microbiome was evaluated in only four studies (14, 17, 26, 31), even though a reduction in skin colonization by *S. aureus* is one of the aims in the use of functional textiles. Beneficial results were seen only with silver, which is understandable given its antimicrobial properties, but no conclusions can be drawn due to the low quality of the supporting evidence. Measures of skin physiology are also important when evaluating skin inflammation. Improvements in TEWL may result from a reduction in skin inflammation associated with a reduction in pruritus and bacterial colonization favored by the use of functional textiles. In our review, we detected conflicting results in the study by Park et al. (27), which showed less or no TEWL improvement in patients with more severe forms of AD.

Although the studies included in this review analyzed different populations, age groups, and degrees of disease severity, only one adverse event—an eczema flare-up—was reported. The event, however, could not be directly linked to the intervention (use of antimicrobial silk fiber), because both treated and untreated areas were affected (29).

The methodological quality of future studies of functional textiles in AD needs be improved to enable similar outcomes to be analyzed across different textiles. The emergence of new compounds may also offer improved effectiveness (42). An appropriate sample size should be calculated according to the evaluated outcomes and type of study design. The possibility of targeting specific AD phenotypes (43) (e.g., *S. aureus* colonization, atopic versus non-atopic, presence or absence of filaggrin gene mutations) may also improve the performance of certain textiles in subgroups of patients. The role of functional textiles in AD needs to be addressed by more studies, with longer follow-up and an improved design.

Conclusions

Based on the low quality of evidence supporting the effectiveness of functional textiles in alleviating symptoms and reducing disease severity in AD, the strength of the recommendation to use these textiles in this setting is weak.

Different textile components are associated with distinct effects; silver-coated fabrics, for example, seem to be more effective at diminishing the severity of lesions, while silk fabrics seem to perform better in terms of alleviating pruritus and other symptoms. Considering the high prevalence of AD, more studies are needed to confirm these data, identify which mechanisms are targeted, and determine how functional textiles contribute to symptom improvement. RCTs with larger sample sizes, longer follow-up periods, new bioactive compounds, and comparisons of similar time interventions and homogeneous study groups in terms of AD severity are needed. The results of such studies could help to identify patients who might benefit most from the use of functional textiles and to determine which textiles are most appropriate in given situations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Grade evidence profile table, assessing the question: should functional textiles be used for atopic dermatitis?

PLOS ONE

RESEARCH ARTICLE

Chitosan Coated Textiles May Improve Atopic Dermatitis Severity by Modulating Skin Staphylococcal Profile: A Randomized Controlled Trial

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Abstract

Background

Atopic dermatitis (AD) patients may benefit from using textiles coated with skin microbiome–modulating compounds. Chitosan, a natural biopolymer with immunomodulatory and antimicrobial properties, has been considered potentially useful.

Objective

This randomized controlled trial assessed the clinical utility of chitosan-coated garment use in AD.

Methods

Of the 102 patients screened, 78 adult and adolescents were randomly allocated to overnight use of chitosan-coated or uncoated cotton long-sleeved pyjama tops and pants for 8 weeks. The primary outcome was change in disease severity assessed by Scoring Atopic dermatitis index (SCORAD). Other outcomes were changes in quality of life, pruritus and sleep loss, days with need for rescue medication, number of flares and controlled weeks, and adverse events. Changes in total staphylococci and *Staphylococcus aureus* skin counts were also assessed. Comparisons were made using analysis of variance supplemented by repeated measures analysis for the primary outcome. Interaction term between time and intervention was used to compare time trends between groups.

Results

Chitosan group improved SCORAD from baseline in 43.8%, (95%CI: 30.9 to 55.9), $P = 0.01$, placebo group in 16.5% (-21.6 to 54.6); $P = 0.02$ with no significant differences between groups; Dermatology Quality of life Index Score significantly improved in chitosan group ($P = 0.02$) and a significant increase of skin Coagulase negative Staphylococci ($P = 0.02$) was seen.

Conclusions

Chitosan coated textiles may impact on disease severity by modulating skin staphylococcal profile. Moreover, a potential effect in quality of life may be considered.

Trial Registration

ClinicalTrials.gov NCT01597817

Introduction

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease with a considerable social and economic burden. In industrialized countries, it has an estimated prevalence of up to 20% in children and 2% in adults [1]. Its pathophysiology is complex and involves skin barrier defects, immunological deregulation, and genetic predisposition [2]. These changes frequently lead to skin colonization with *Staphylococcus aureus*, which is able to produce virulence factors that perpetuate inflammation, even in normal-appearing skin [3]. Disease management demands an integrated approach, aimed not only at controlling skin inflammation and ensuring hydration, but also at regulating the skin microbiome [4–6].

While several recent studies have reported the utility of functional textiles with antimicrobial and antipruritic properties in AD [7, 8], a recent systematic review and meta-analysis by our group found that the recommendation for its use was weak due to the low quality of supporting evidence [9]. These results underscored the need for studies with improved methodology and new compounds. Chitosan, a biopolymer [10], has been considered a promising candidate for use in AD due to its with repair and antiseptic properties [11–13]. Chitosan-coated fabrics with proven inhibitory activity against *S. aureus* were considered potentially useful in AD management, but their clinical utility on a real life setting has never been studied.

This randomized controlled clinical trial assessed the clinical utility of chitosan-coated garments in AD patients.

Methods

This is a randomized, double-blind, placebo-controlled, single-center trial. Fig 1 shows the flow of participants. Trial registrations: ClinicalTrials.gov Identifier: NCT01597817. Protocol Registration and Results System account administration delay in releasing the record due to informatics issues caused that the trial was registered after enrolment of participants had started. The authors confirm that all ongoing and related trials for this intervention are registered. Ethics committee approved the study at 6th September 2011, patients recruitment and follow up occurred between December 2011 and June 2012.

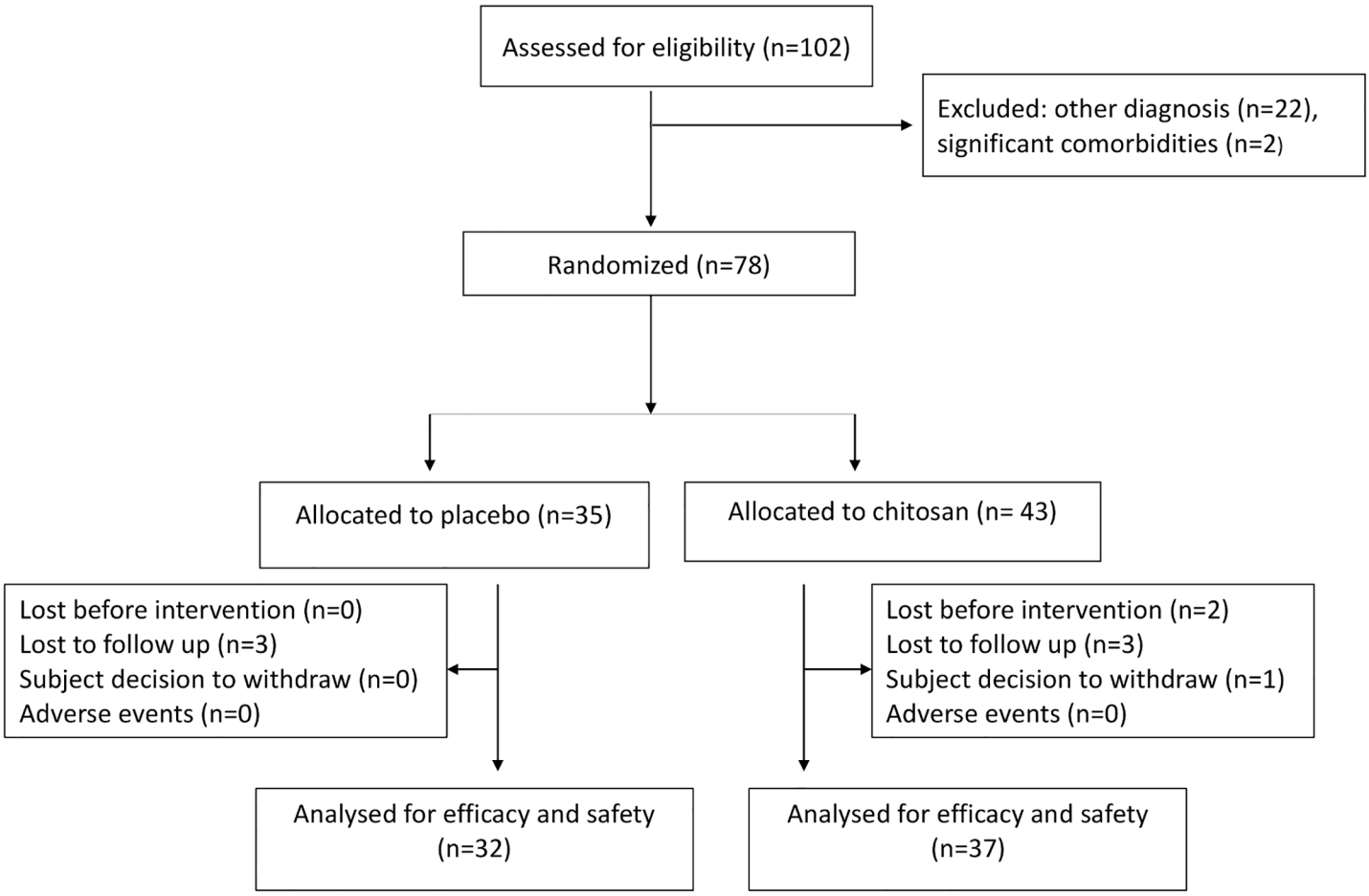

Fig 1. Flow chart of participants through the study.

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Recruitment

Subjects were invited to participate in the trial during hospital visits, through trial posters on bulletin boards in hospitals, newspaper and Internet advertisements.

Inclusion and exclusion criteria

Subjects older than 12 years with a diagnosis of AD [14] were eligible for participation following provision of written informed consent. Excluded were patients with severe skin disease other than AD (e.g., psoriasis); secondary infections; major systemic diseases; women who were pregnant and subjects unable to comply with study and follow-up procedures.

Patients who met any of the following criteria were withdrawn from the study: use of topical or systemic antibiotics during the study; withdrawal of consent; detection of significant protocol violations; and investigator's decision to withdraw the patient due to adverse effects such as skin infections.

Sample size

Sample size calculations were performed to determine the number of participants needed to detect effect sizes based on minimal clinically important differences in the SCORAD index.

Results showed that 42 patients were needed in this two-treatment parallel-design study to detect a treatment difference with a two-sided 0.05 significance level and a probability of 81% if the true difference in SCORAD between interventions was 8.7 units [15].

Randomization, allocation and blinding

Subjects were randomly assigned to one of two interventions through computer-generated random numbers. The randomization was performed by an independent researcher;the randomization table and intervention codes were kept by the independent researcher in an opaque sealed envelope up to completion of data analysis. A study nurse established phone contact with the independent researcher, who informed the nurse which treatment package was to be assigned to which patient.

A hundred and two patients were assessed for eligibility; twenty-four were excluded because they did not meet inclusion criteria: 22 because the medical diagnosis of AD was not confirmed by the investigation team and two because of significant comorbidities (multiple sclerosis and diabetes mellitus type 1). Seventy-eight were randomized: thirty five to placebo and forty three to chitosan groups. In chitosan group two patients were lost before receiving the intervention and one patient decided to withdraw because of disease progression. In both groups three patients were lost to follow up due to impossibility to attain medical visits (Fig1).

Treatment protocol and intervention

The study consisted of a 2-week run-in period and an intervention period of 8 weeks (S1 Table). Eligibility to participate was determined at the screening visit. At the end of the run-in period, the patients were examined by the same physician as in the first visit and those with a change in SCORAD of below 10% with respect to baseline were considered eligible for randomization. Participants were randomized to receive either an uncoated pair of cotton pyjamas or a pair of cotton pyjamas coated with chitosan (ChitoClear CG-800). The pyjamas, placed in a sealed plastic package, consisted of a long-sleeved top and long pants to be worn at night for the duration of the study. Both pyjamas were made of 100% organic cotton, without dyes or preservatives, and were visually indistinguishable from each other. The *in vitro* antibacterial activity of the chitosan-coated textile was shown to persist after 30 washing cycles [16] and washing durability was studied through washing assays at 40°C [16].

Outcomes and definitions

The primary efficacy outcome measure was mean relative and absolute change in disease severity after the intervention assessed by SCORAD [14]. The SCORAD index combines objective items reflecting disease extent, intensity and subjective items (pruritus and sleep loss) evaluated by the patient on a 10-point visual analog scale (VAS), where 0 indicates no pruritus or sleep loss and 10 indicates the worst possible pruritus and sleep loss. The total possible score ranges from 0 to 103.

Secondary outcome measures were number of patients with a minimal clinically important difference in SCORAD post-intervention; mean change in quality of life score; changes in daily pruritus and sleep loss scores; need for rescue medication; number of flares; number of totally controlled weeks (TCWs) and well-controlled weeks (WCWs); and number and severity of adverse events during the 8-week study period. Microbiological outcome measures were mean changes in colony forming units (CFUs) per 100 cm^2 of total staphylococci (*S. aureus* plus coagulase negative staphylococcus species) and *S. aureus* isolates.

Patients were characterized according to age, gender, current medication, personal history of atopy, self-reported medical diagnosis of asthma, disease duration, and disease severity. The

SCORAD index was used to classify AD as mild (score $\leq$15), moderate (16–39), or severe (>40) [17]. During the baseline and final visits, participants were asked to complete the Portuguese version of the Dermatology Life Quality Index (DLQI) or, if they were younger than 16 years, the children´s version of the questionnaire. Both questionnaires have been translated and validated for use in the Portuguese population [18, 19]. DLQI scores are interpreted as no effect on the patient's life (score of 0–1), a small effect (2–5), a moderate effect (6–10), a very large effect (11–20), and an extremely large effect (21–30) [20, 21].

Participants recorded and scored daily symptoms of pruritus and sleep loss according to the 10-point VAS, and registered all medication use during the study period. Rescue medication was defined as any treatment, other than emollient, applied in response to disease worsening (i.e. escalation of treatment). A flare was defined as an episode requiring rescue medication for 3 or more consecutive days; a TCW week as a pruritus score of above 4; and a WCW as a 7-day period with need for rescue treatment or with a sleep loss or pruritus score of above 4 for no more than 2 days. [22].

Microbiological assays

The microbiological profile was assessed by determination of viable cell numbers of total staphylococci and *S. aureus* in five regions: the right and left brachial crease, right and left popliteal crease, interscapular region. The regions were assessed by sampling a 25-cm^2 area of skin with a sterile cotton swab dipped in sterile saline solution. Samples were kept refrigerated at 4°C and were processed within a maximum of 2 hours of sampling. They were decimally diluted and plated in Mannitol Salt agar (MSA; Lab Mspread plate) and Baird-Parker agar (BPA; Lab M, Lancashire, UK) using the spread plate technique. After incubation, the colonies were counted, using MSA for total staphylococcal counts and BPA for *S. aureus* counts, the respective Colony Forming units /100 cm^2 were determined.

Adverse events

Patients were asked to inform the research team of any possible adverse events that occurred during the 8-week study period. Adverse events were classified as mild if they were easily tolerated by the patient; moderate if they interrupted the individual's usual activities; and severe if they were potentially life-threatening. The principal investigator classified adverse events as not, possibly, probably, or definitely related to treatment.

Statistical analysis

All efficacy outcomes were analyzed using intent-to-treat populations based on the treatment group assigned at randomization.

Analysis of variance (ANOVA) supplemented by a repeated measures analysis was used for the primary outcome. A mixed effects models with random intercept and time slope by individual were used to estimate the interaction term to compare time trends between groups for number of days per week with need for rescue medication and daily symptoms. Chi-squared, Fisher exact and McNemar test were used for secondary outcomes; Wilcoxon signed rank test and Mann Whitney test for non-parametric analysis; *t* test for parametric analysis. All analyses, summaries, and listings were performed with SPSS software, version 20.0.

Ethics

The university and hospital ethics committees approved the present study (ClinicalTrials.gov Identifier: NCT01597817). Written informed consent was obtained from each participant and from parents, caretakers, or guardians on behalf of the minors/children prior to enrolment.

The trial was performed in compliance with the Declaration of Helsinki and according to good clinical practice.

Results

Patients characteristics

No major imbalances were found in the baseline characteristics of the individuals included in the placebo and chitosan groups: most patients were adult, with AD for more than 10 years, more than half were female, the majority were atopic and had self reported previous history of asthma (Table 1). Oral antihistamines and topical steroids were used by most patients, almost half had been prescribed at least once oral steroids in the last year and a systemic immunosuppressor such as cyclopsorin in 17% overall. Similar proportion of participants with mild (2 versus 5), moderate (19 versus 14) and severe (22 versus 16) AD occurred respectively in chitosan and placebo intervened groups.

Efficacy and tolerability. After the 8-week intervention period there was a significant improvement in SCORAD from baseline for both the chitosan group and the placebo group (improvement of 43.8%, 95% CI: 30.9 to 55.9; $P = 0.01$ vs. 16.5%, 95% CI: -21.6 to 54.6; $P = 0.02$). The respective absolute reductions in SCORAD scores were from 44.2 (95% CI: 34.5 to 53.9) to 29.4 (95% CI: 21.4 to 37.4) and 41.4 (95% CI: 34.3 to 48.6) to 25.7 (95% CI: 18.3 to 33.1); (Fig 2). No significant differences were observed between groups for changes in SCORAD.

Table 1. Baseline characteristics of atopic dermatitis patients by chitosan intervention group (*N* = 43) and placebo group (*N* = 35).

	Chitosan	Placebo	P-value
Age, y	23 (19–34)	26 (18–31)	0.61§
Female, *n* (%)	23 (53)	21 (60)	0.86*
Disease duration, y	18 (10–24)	12.0 (6–20)	0.31§
SCORAD (0–103)	44 (25–52)	38 (22–65)	0.72§
Current medication			
Antihistamines, *n* (%)	36 (84)	32 (91)	0.50*
Topical corticosteroids, *n* (%)	37 (86)	27 (77)	0.18*
Oral corticosteroids, *n* (%)	15 (35)	16 (46)	0.58*
Calcineurin inhibitors, *n* (%)	12 (28)	16 (46)	0.18*
Oral immunsupressors, *n* (%)	9 (21)	4 (11)	0.13ε
Diary scores			
Pruritus (0–10)	4 (2–4)	3 (2–5)	0.92§
Sleep loss (0–10)	2 (1–4)	1 (0–3)	0.31§
DLQI score	7 (5–12)	7 (5–12)	0.93§
Atopic, *n* (%)	29 (70)	21 (60)	0.29*
Asthmatic, *n* (%)	21 (49)	18 (51)	0.69*

DLQI, Dermatology Life Quality Index. Results are presented as median (interquartile range) unless stated otherwise.

§ Mann Whitney test.

* Chi-squared test

ε Fisher exact test.

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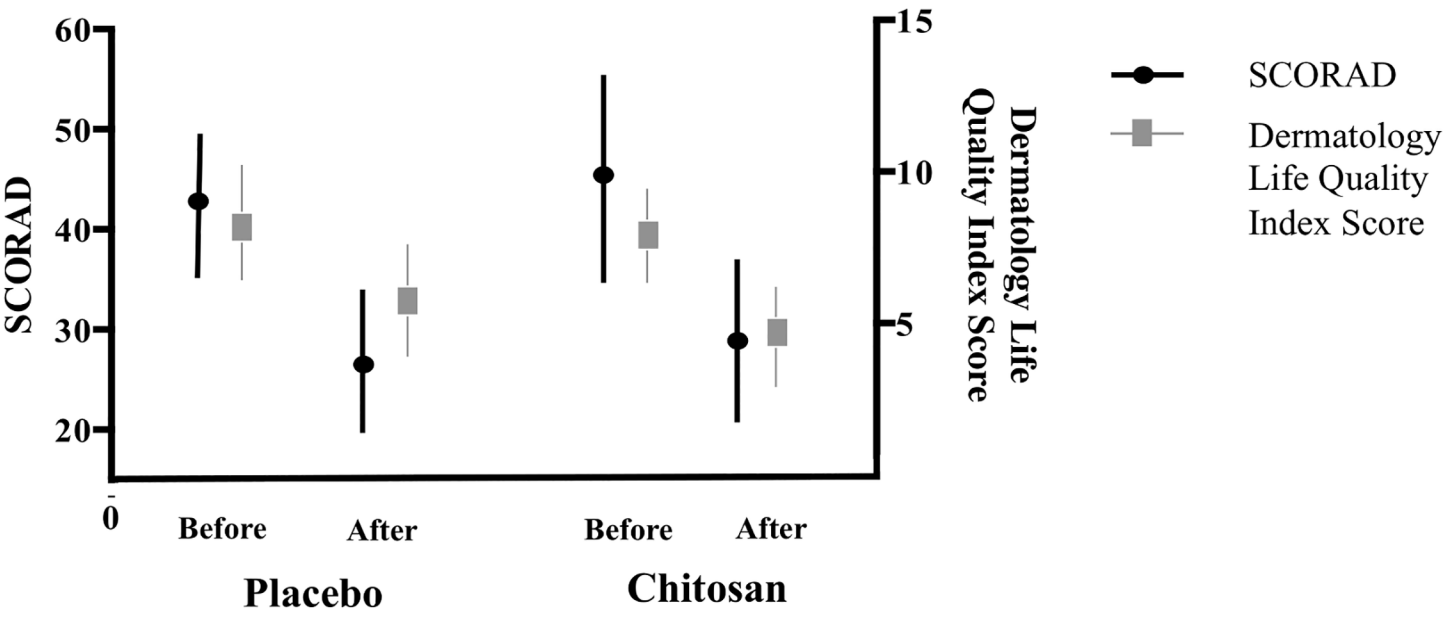

Fig 2. Mean SCORAD and Dermatology Life Quality Index scores (95% CI) in chitosan and placebo groups before and after intervention. CI-confidence interval.

doi:10.1371/journal.pone.0142844.g002

The improvement in DLQI scores from baseline was 36% (95% CI: 23.5 to 48.1) in the chitosan group (8.0 [9.3–6.7] to 4.8 [6.2–3.4], $P = 0.02$) and 25% (95% CI: 6.0–44.1) in the placebo group (8.3 [10.4–6.3] to 5.6 [7.7–3.5], $P = 0.28$) (Fig 2). There were no significant differences between both groups. The proportion of individuals with a clinically meaningful improvement in SCORAD was 25 (67%) in the chitosan group and 20 (63%) in the placebo group. No significant effect was observed either on daily pruritus or sleep loss scores (Fig 3 and S2 Table), need for rescue medication, or number of flares or totally controlled weeks and well controlled weeks (Table 2).

Most patients had identification of Staphylococci species in at least one sampled region with no significant changes after the intervention or for changes between groups (Table 3). There a was a decrease in the percentage of patients with identification of S.aureus from 68% to 55% in chitosan group in contrast with an increase in placebo group (from 53% to 64%) that did not reach statistical significance (Table 3). The mean proportion of S.aureus counts versus total staphylococcal counts showed no significant differences after intervention for both groups on the five sample regions (right arm, left arm, right leg, left leg, neck) (Table 3) neither when considering all regions (Fig 4). When considering total bacterial counts there was a significant increase of the mean total staphylococcal count in the chitosan group ($P = 0.02$), with no other differences (Fig 5).

The chitosan-coated pyjamas were well tolerated. One patient in the chitosan group decided to withdraw at week 4 due to an AD flare, but no causal link was established.

Discussion

In this randomized controlled trial chitosan coated textiles, used for 8 weeks, were associated with a non-significant trend of disease severity improvement. Moreover, this effect was related with a significant increase of skin coagulase negative Staphylococci.

Our study has some limitations. First, since this is a pilot study the number of participants and outcomes assessed may have been not sufficient to detect significant differences. However,

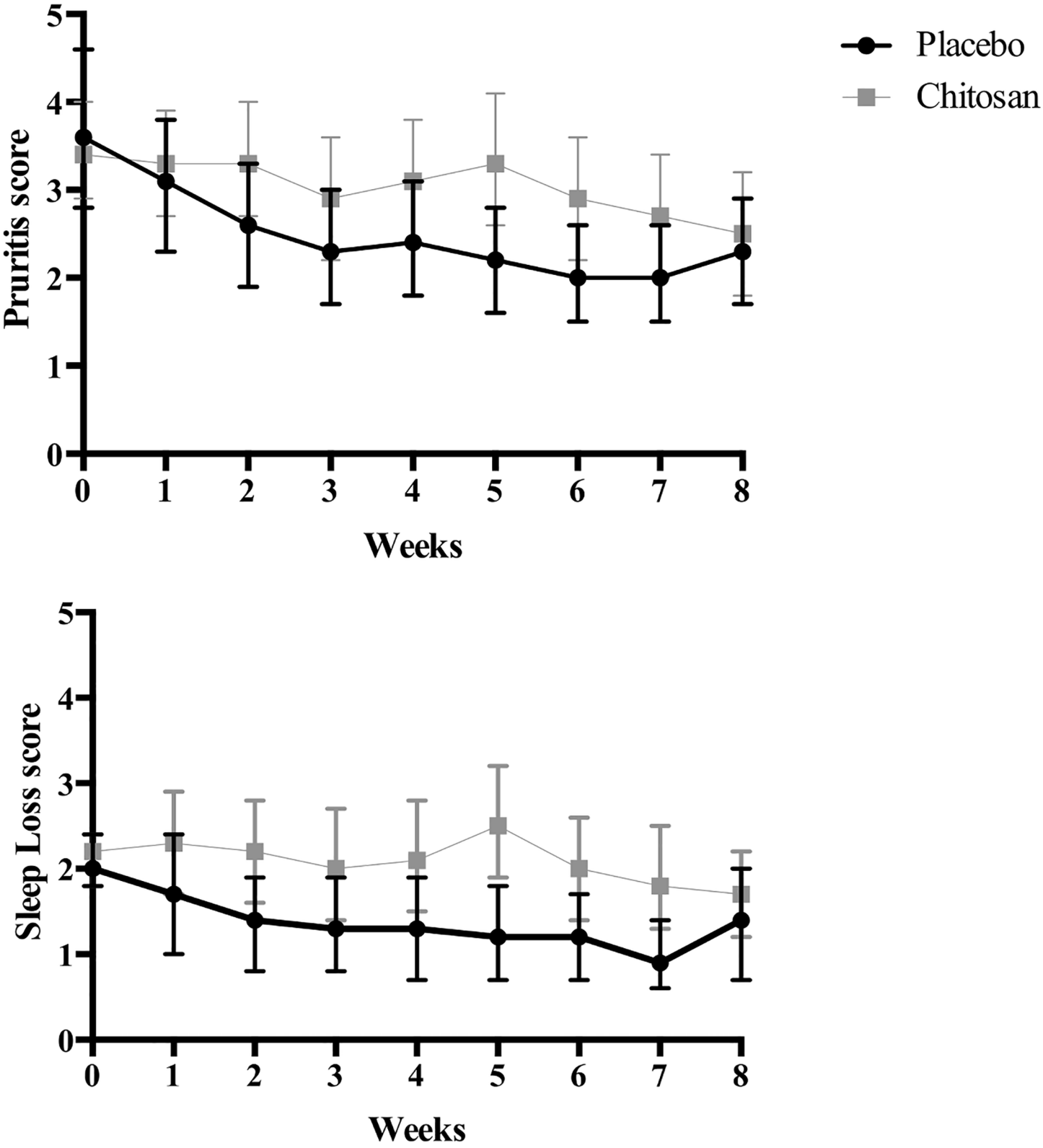

Fig 3. Mean (95% CI) weekly pruritus and sleep loss scores in chitosan and placebo groups throughout the intervention period. CI-confidence interval.

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Table 2. Differences in efficacy outcomes in chitosan and placebo groups after intervention.

	Chitosan	Placebo	P-value for difference[§]
Rescue medication, days	2.0 (0.0–8.3)	5.0 (0.0–15.5)	0.82
Flares	0.0 (0.0–1.0)	0.0 (0.0–1.0)	0.73
Totally controlled weeks	4.0 (0.8–7.0)	4.5 (1.8–8.0)	0.43
Well controlled weeks	1.5 (0.8–3.0)	2.0 (0.0–3.0)	0.82
Uncontrolled weeks	1.0 (0.0–4.3)	1.0 (0.0–5.0)	0.94

Median (interquartile range)

[§] Mann Whitney test.

Rescue medication defined as any treatment, other than emollient, that was applied in response to a worsening of the disease, corresponding to dosing up treatment; a flare as need of rescue medication for three or more consecutive days; a totally controlled week as a seven-day period without need of rescue treatment and without any days of sleep loss or pruritus score above 4; a well controlled week if rescue treatment and sleep loss or pruritus score above 4 occurred for no more than 2 days, and any other week that did not correspond to the previous definitions of totally and well controlled weeks was classified as no controlled;

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based on previously published minimally clinically important differences for SCORAD, the study was designed to be sufficiently powered to detect meaningful differences. However, post hoc analysis analysing the high range of confidence limits in the control group versus the active one suggested this may have not been the case. Although we only used validated outcomes in line with recently published recommendations [23], DLQI did not fit Rasch analysis in previous studies [24] and its children´s version has not been tested till now. Nevertheless it has been previously validated in Portuguese population[19] and in our study we found an intraclass correlation coefficient in placebo group of 0.73 signifying a good reproducibility. Second, the study participants were adolescents and adults with long-standing atopic dermatitis and there would probably be a greater likelihood of detecting clinically significant improvement in adults with more severe disease. Thirdly, because no *a priori* data exist on the duration of the intervention and its *in vivo* effects, we cannot rule out that longer skin contact with chitosan may have elicited a more pronounced effect. However, the participants were instructed to wear the

Table 3. Skin microbiological profile in chitosan and placebo groups before and after intervention.

	Chitosan			Placebo			Chitosan vs Placebo
	Before (*N* = 38)	After (*N* = 34)	*P*-value	Before (*N* = 30)	After (*N* = 28)	*P*-value	*P*-value
Staphylococci +, *n* (%) of patients	34 (85)	30 (75)	0. 71 [P]	26 (87)	23 (82)	0.92 [P]	0.68[Φ]
S. aureus +, n (%) of patients	27 (68)	22 (55)	0.92 [P]	18 (53)	18 (64)	0.72 [P]	0.69.[Φ]
% CFU *S. aureus*/total staphylococci							
Right arm	58 (14–74)	55 (18–68)	0.43*	71 (38–94)	81 (31–96)	0.21*	0.14[§]
Left arm	62 (12–68)	61 (12–77)	0.94*	65 (38–81)	67 (39–70)	0.42*	0.34 [§]
Right leg	66 (18–73)	65 (13–76)	0.32*	68 (22–78)	67 (22–89)	0.52*	0.92 [§]
Left leg	70 (18–82)	69 (25–77)	0.91*	69 (24–78)	71 (36–88)	0.83*	0.73 [§]
Neck	58 (22–71)	42 (22–61)	0.11*	74 (21–80)	76 (29–92)	0.34*	0.93 [§]

CFU, colony-forming units. Median (interquartile range) unless stated otherwise.

[P] McNemar test

[Φ] Chi-squared test

* Wilcoxon signed rank test

[§] Mann Whitney test.

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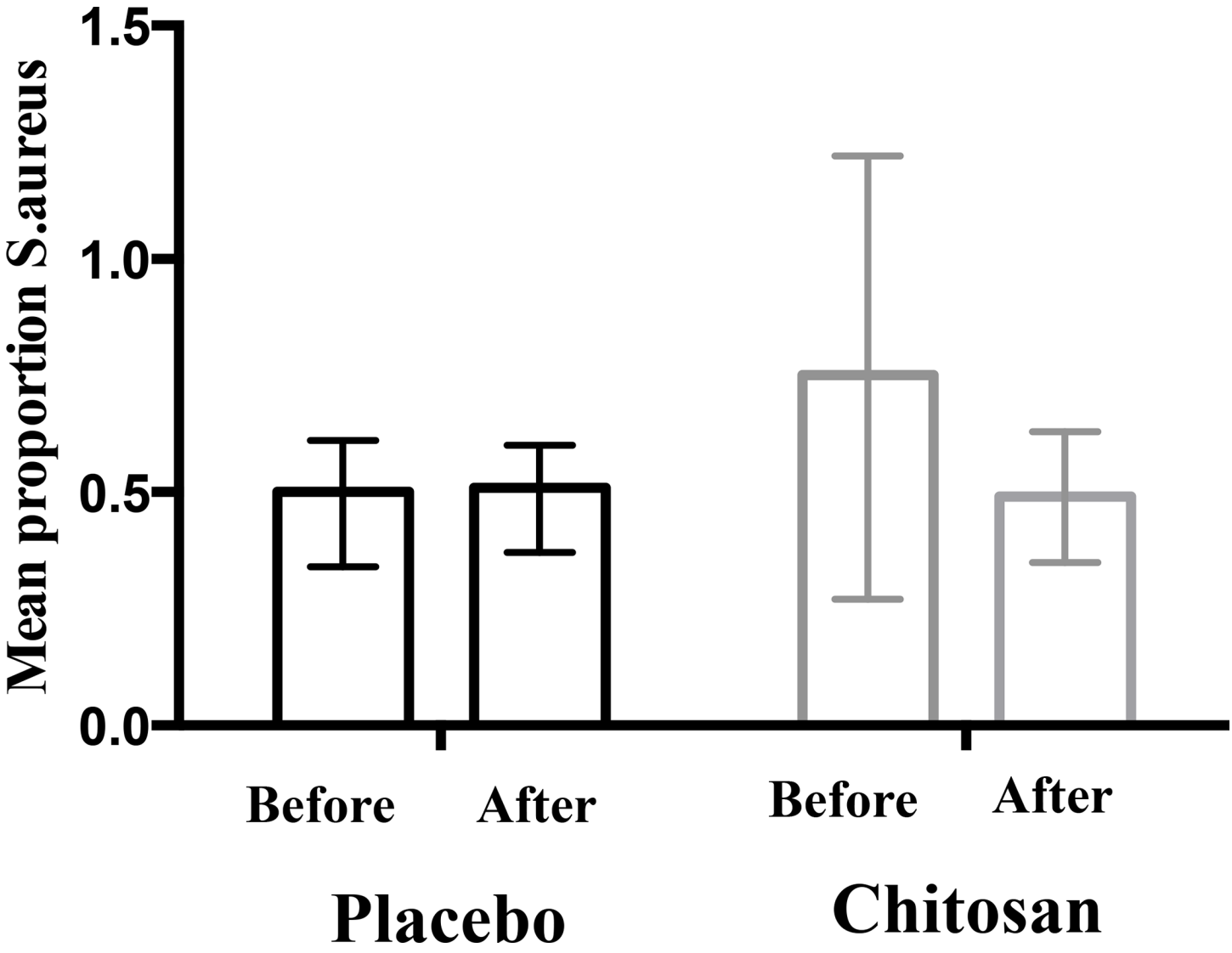

Fig 4. Mean (95% CI) *Staphylococcus aureus* colony forming units in all regions as proportion of total staphylococcal counts before and after intervention in placebo and chitosan groups. CI-confidence interval.

doi:10.1371/journal.pone.0142844.g004

pyjamas every night for the duration of the study, as we wished to target a critical period. Finally, the fact that the patients were allowed to use rescue medication may have influenced the effect of the intervention. However, this was corrected for in the mixed effects model and the effect on clinical outcome analyses should therefore be minimal. This is the first trial to evaluate the utility of a biopolymer in patients with AD and, so far, it is the largest study of functional textiles. Another innovative aspect was the evaluation of other staphylococcal species than *S.aureus* [25].

Chitosan has exhibited skin repair potential in wounds and antimicrobial action in diverse medical fields [26–29], explaining why chitosan could potentially improve disease severity in patients prone to non-commensal bacteria colonization and skin barrier impairment. In the present study, chitosan-coated garments had no effect on the skin *S.aureus* counts but surprisingly, we observed in the chitosan group an increase in total staphylococci counts independently of *S. aureus*, corresponding to coagulase negative staphylococci species (CNS). The

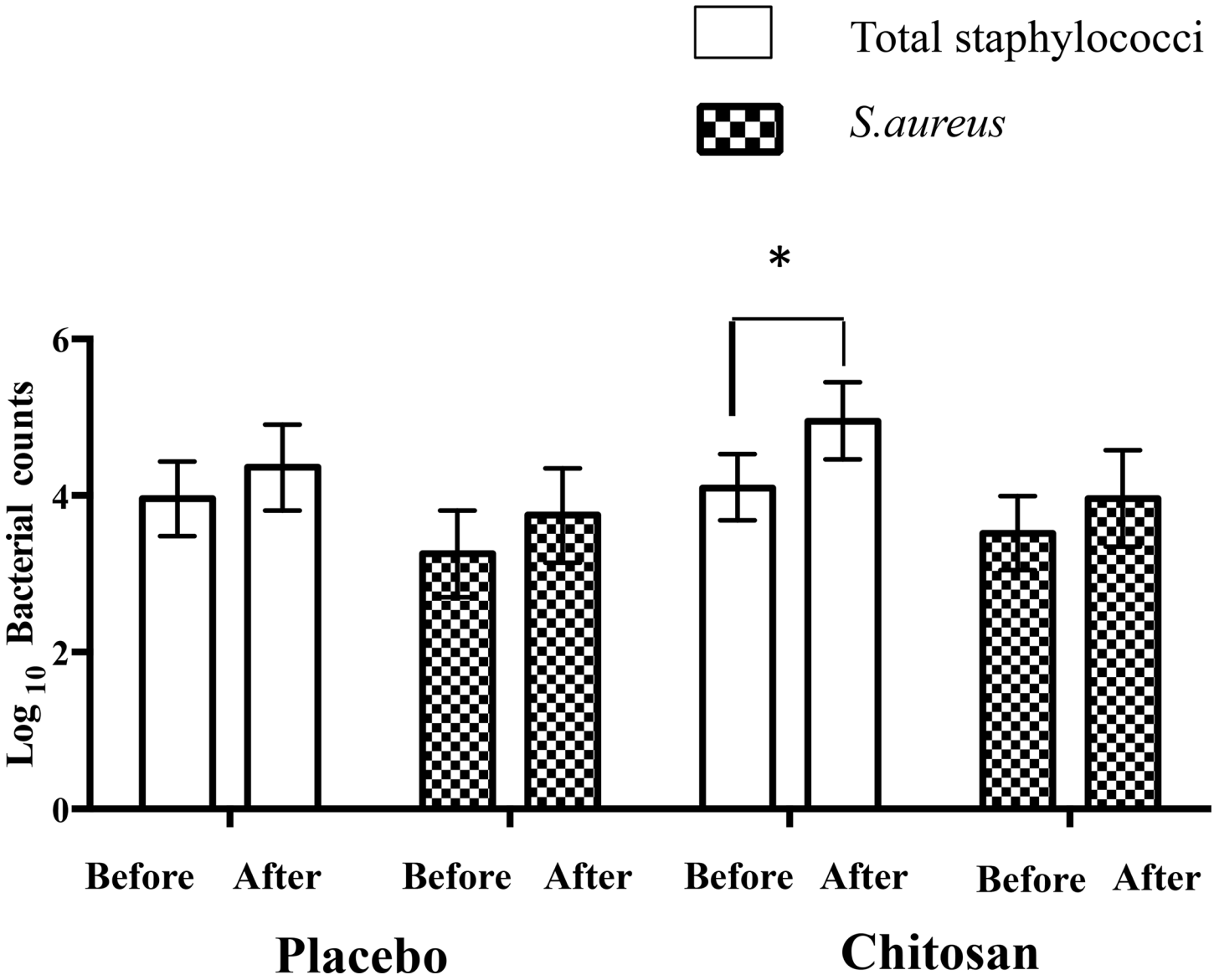

Fig 5. Mean (95% CI) Log10 total staphylococci and Log10 Staphylococcus aureus counts for all regions sampled in chitosan and placebo groups before and after intervention. CI-confidence interval *$P = 0.01$, Wilcoxon signed rank test.

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increase of CNS on the skin of AD patients has been already reported eliciting different explanations for this fact: some authors argue that it may be the result of a mutualistic relationship or represent a compensatory or antagonistic mechanism to control *S.aureus* [30]. Our data supports the hypothesis that chitosan may had exerted a specific inhibitory effect upon *S. aureus*, allowing the proliferation of other staphylococcal species. Nevertheless, the clinical significance of this observation is exploratory.

The observed placebo effect on disease severity may possibly be due to the improved skin comfort provided by the long-sleeved organic cotton pyjamas used, and/or to the patients' enthusiasm about participating in a clinical trial with a new product.

The significant improvement on quality of life with chitosan treatment was probably related to reduction in AD severity in this group. Considering that sample size was calculated to detect

changes in SCORAD index, we cannot exclude that more patients were needed to elicit a more pronounced effect on this outcome.

The intervention was well tolerated over the 8-week study period. There was one moderate adverse event, deemed to be unrelated to treatment, in the chitosan group. Safety of functional textiles is a controversial issue since some authors have claimed that the use of antimicrobial compounds could remove bacteria from the skin surface and pave the way for invasion by pathogenic bacteria, such as methicillin-resistant *S. aureus* [31].

Atopic dermatitis is a complex disease that requires a multidimensional treatment approach. The possibility of modulating the skin microbiome, namely its staphylococcal community, which has long been recognized as one of the main determinants of skin inflammation, is an appealing strategy. The use of functional textiles is also appealing because of their potential action targeting the skin surface and their favourable safety profile and convenience of use. Results from our randomized controlled trial showed that chitosan coated textiles may impact on disease severity by modulating skin staphylococcal profile. Moreover, a potential effect in quality of life may be considered.

Supporting Information

S1 CONSORT Checklist. Consort Checklist.
(DOCX)

S1 PROTOCOL. Ethic Committee protocol.
(DOCX)

S1 Table. Study schedule D, day; W, week.
(DOCX)

S2 Table. Mixed effects model comparing time trends between chitosan and placebo groups. SD-standard deviation.
(DOCX)

Author Contributions

Conceived and designed the experiments: CL DS LD AM. Performed the experiments: CL JS FT AD OC DS. Analyzed the data: CL JS FT OS DS MS LD AM. Contributed reagents/materials/analysis tools: MS OS. Wrote the paper: CL FT OS MS MP DS AM LD.

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Chitosan coated textiles increase serum eosinophil cationic protein but not specific IgE in atopic dermatitis patients

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Abstract:

Chitosan coated textiles have been increasingly used in Dermatology field due to its previously reported antimicrobial, immunostimulatory and repairing activity in skin burns and wounds.[1-6] Atopic dermatitis (AD) is an inflammatory skin disease occurring most often in children but that can persist into adulthood when it tends to be more severe[7]. It is characterized by intensely pruritic inflammatory lesions associated with an increased predisposition to colonization with skin bacteria *Staphylococcus aureus* and epidermal barrier impairment. It is related with immunological deregulation characterized by higher levels of serum specific Immunoglobulin E (IgE) against *Staphylococcus aureus* that can perpetuate skin inflammation[8] but the role of eosinophil degradations products as eosinophilic cationic protein (ECP) in AD is controversial, since ECP has antibacterial activity that may exert protective properties[9, 10]. Chitosan coated textiles have been considered potentially useful in AD management but its impact on eosinophilic and IgE mediated serum biomarkers is unknown.

This randomized, double blind, placebo-controlled, single center trial aims to assess the effect of chitosan-coated garments use in immunoallergic serum biomarkers of AD patients.

Patients were invited to participate trough media advertisements; subjects older than 12 years, with confirmed diagnosis of AD [11] that provided informed consent were included. Participants with severe skin disease other than AD, secondary infection or any major systemic disease were excluded. A total of 62 patients were needed to detect a treatment difference at a two-sided 0.05 significance level with a probability of 81 %. Patients were randomized to receive a pair of cotton (placebo) or β (1-4) D-glucosamina/N-acetyl-D-glucosamina with 76% deacetylation (chitosan) coated cotton long sleeved T-shirt and pants to be used as pyjama during the night for 8 weeks. For chitosan fabric preparation cotton fabric was completely immersed in a solution containing 1% chitosan (ca. 600 KDa) and Glyoxal as cross linking agent (2.5%). Impregnation was achieved by the pad-dry-cure method. The pick up was determined by weighting the fabric before and after the impregnation and found to be 79%. The fabric was allowed to dry at 100ª C for 4 min after which it was thermofixed at 140ª C for 4 min. The chitosan solutions were previously prepared in 1% (V/V) acetic acid and allowed to dissolve for 24 h at 50ºC; the pH was then adjusted to 5.6-5.8 with NaOH (10 M). It was previously shown that chitosan coated textiles followed ISO 20743:2007, maintaining its properties after 30 washing cycles[12].

Both patients and investigators were blinded to intervention. Changes in serum total IgE, eosinophil cationic protein and specific IgE to a mixture of inhalant allergens (Phadiatop™), *S. aureus* enterotoxins A, B, C, TSST and *Malassezia spp* (ImmunoCap ™) were determined before and after intervention. Hospital Ethics Committee approved the study.

Of the 102-screened subjects, 22 were excluded due to other diagnosis, 2 because of significant comorbidities; 43 received placebo and 35 chitosan garments (Table 1). Parametric and non parametric tests were used as appropriate (SPSS, version 20.0).

A significant difference in changes in serum level of ECP was observed between placebo and chitosan group ($p=0.025$). No further differences existed (Table 2).

It has been previously described that chitosan accelerates migration of polymorphonucleares to wound areas, secreting inflammatory mediators such as TNF – α and interleukin -1.[4] These effects seems to protect eosinophils from apoptosis under inflammatory conditions in *in vivo* mouse models.[13] Since ECP is an indirect marker of eosinophil degranulation, we may hypothesize that chitosan textiles promoted eosinophil survival and activation contributing to increase in serum ECP. The clinical implication of this result is unknown.

Our study has a few limitations. First, because no *a priori* data exists, we cannot rule out that a more prolonged skin contact with chitosan or higher number of included subjects may have elicited a significant effect on IgE markers. Secondly, since patients were included regardless of their atopy status, the selection of specific AD phenotypes could have determined different results.

Importantly, this the first study addressing the impact of chitosan textiles in serum immunoallergic markers of AD patients. Our findings suggest that overnight use for 8 weeks of chitosan textiles is associated with increased serum ECP but not IgE mediated allergic inflammation. Further studies are needed to evaluate the clinical relevance of our data.

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Table 1. Baseline characteristics of atopic dermatitis patients in chitosan (N = 43) and placebo groups (N = 35)

	Chitosan	Placebo	p
Age, y	23.0 (19)	26 (15)	0.611§
Female, n (%)	23 (53)	21 (60)	0.923*
Disease duration, y	18.0 (17)	12.0 (15)	0.312§
SCORAD (0-103)	44.0 (32)	37.8 (38)	0.724§
Atopic, n (%)	29 (70)	21 (60)	0.331*
Asthmatic, n (%)	21 (49)	18 (51)	0.922*

Results are presented as median (interquartile range) unless stated otherwise *Chi-squared exact test. §Non-parametric Mann-Whitney U test.

Table 2. Diferences in immunoallergic outcomes according to intervention

	Chitosan (n=43)			Placebo (n=35)			Chitosan vs. Placebo
	Before	After	p-value¶	Before	After	p-value¶	p-value
Total IgE, UI/ml	5215 (2135 to 8284)	3591 (1437 to 5746)	0.012	2238 (790 to 3686)	1886 (540 to 3232)	0.001	0.72*
Phadiatop, KUA/L	635 (255 to 1014)	475 (209 to 742)	0.001	309 (142 to 475)	263(107 to 419)	0.001	0.64§
Specific IgE, KUA/L							
Enterotoxin A	5.6 (-1.5 to 12.9)	3.3 (-0.16 to 6.8)	0.8	1.71 (o.57 to 2.8)	1.7 (0.4 to 2.9)	0.62	0.52 §
Enterotoxin B	2.95 (0.6 to 5.4)	3.7 (-0.5 to 7.8)	0.14	1.78 (0.6 to 3.01)	1.6 (0.1 to 3.0)	0.94	0.31 §
Enterotoxin C	3.2 (1.4 to 5.0)	2.9 (1.7 to 4.1)	0.93	3.3 (1.8 to 4.7)	4.6 (0.7 to 8.4)	0.95	0.87§
Enterotoxin TSST	2.68 (-0.1 to to 5.5)	4.3 (-1.7 to 10.5)	0.12	0.96 (0.4 to 1.53)	1.2 (0.5 to 2.0)	0.91	0.27§
Malassezia furfur	8.6 (1.6 to 15.6)	12.7 (2.9 to 22.4)	0.17	8.4 (2.7 to 14.1)	14.9 (3.8 to 25.9)	0.04	0.1§
Eosinophil cationic protein,	28.6 (23.2 to 34.1)	41.5 (28.9 to 54.1)	0.001	33.8 (23.6 to 44.0)	28.6 (23.2 to 34.1)	0.63	**0.025***

Mean (95%CI) unless stated otherwise; ¶ wilcoxon Ranked sign test *ANOVA test with Baseline values as covariate, intervention as fixed effects **§** Mann-Whitney U test for independent groups. TSST